CLH report

Proposal for Harmonised Classification and Labelling

**Based on Regulation (EC) No 1272/2008 (CLP Regulation),
Annex VI, Part 2**

Substance Name: SULFOXAFLOR

EC Number: **Not available**

CAS Number: **946578-00-3**

Index Number:

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Part A.

Part A.

1 PROPOSAL FOR HARMONISED CLASSIFICATION AND LABELLING

1.1 Substance

Table 1: Substance identity

Substance name:	*Sulfoxaflor* *Synonyms XDE-208/XR 208*
EC number:	*Not available*
CAS number:	*946578-00-3*
Annex VI Index number:	*New active substance - No current entry*
Degree of purity:	*95 % w/w*
Impurities:	*Confidential data*

1.2 Harmonised classification and labelling proposal

Table 2: The current Annex VI entry and the proposed harmonised classification

	CLP Regulation	**Directive 67/548/EEC (Dangerous Substances Directive; DSD)**
Current entry in Annex VI, CLP Regulation	New active substance - No current entry	New active substance - No current entry
Current proposal for consideration by RAC	Acute Tox 4; H302 Aquatic Acute 1; H400 Aquatic Chronic 1; H410	Xn; R22 N; R50/53
Resulting harmonised classification (future entry in Annex VI, CLP Regulation)	Acute Tox 4; H302 Aquatic Acute 1 H400 Acute M-factor 1 Aquatic Chronic 1 H410 Chronic M-factor 1	Xn; R22 N ; R50/53 Specific concentration limits: Concentration classification C≥25% N: R50/53; 2.5%≤C<25% N: R51/53; 0.25%≤C<2.5%: R52/53

1.3 Proposed harmonised classification and labelling based on CLP Regulation and/or DSD criteria

Table 3: Proposed classification according to the CLP Regulation

CLP Annex I ref	Hazard class	Proposed classification	Proposed SCLs and/or M-factors	Current classification [1)]	Reason for no classification [2)]
2.1.	Explosives	None			No classification warranted based on the study
2.2.	Flammable gases	None			Substance is not a gas
2.3.	Flammable aerosols	None			Substance is not an aerosol
2.4.	Oxidising gases	None			Substance is not a gas
2.5.	Gases under pressure	None			Substance is not a gas
2.6.	Flammable liquids	None			Substance is not a liquid
2.7.	Flammable solids	None			No classification warranted based on the study
2.8.	Self-reactive substances and mixtures	None			Not evaluated
2.9.	Pyrophoric liquids	None			Substance is not a liquid
2.10.	Pyrophoric solids	None			Not evaluated
2.11.	Self-heating substances and mixtures	None			No classification warranted based on the study
2.12.	Substances and mixtures which in contact with water emit flammable gases	None			Not evaluated
2.13.	Oxidising liquids	None			Substance is not a liquid
2.14.	Oxidising solids	None			No classification warranted based on the study
2.15.	Organic peroxides	None			Substance is not a peroxide
2.16.	Substance and mixtures corrosive to metals	None			Not evaluated
3.1.	Acute toxicity - oral	Acute Tox 4; H302	-	-	-
	Acute toxicity - dermal	No classification	-	-	Conclusive, but not sufficient for classification
	Acute toxicity - inhalation	No classification	-	-	Conclusive, but not sufficient for

					classification
3.2.	Skin corrosion / irritation	No classification	-	-	Conclusive, but not sufficient for classification
3.3.	Serious eye damage / eye irritation	No classification	-	-	Conclusive, but not sufficient for classification
3.4.	Respiratory sensitisation	-	-	-	No data
3.4.	Skin sensitisation	No classification	-	-	Conclusive, but not sufficient for classification
3.5.	Germ cell mutagenicity	No classification	-	-	Conclusive, but not sufficient for classification
3.6.	Carcinogenicity	No classification			Conclusive, but not sufficient for classification
3.7.	Reproductive toxicity	No classification	-	-	Conclusive, but not sufficient for classification
3.8.	Specific target organ toxicity –single exposure	No classification	-	-	Conclusive, but not sufficient for classification
3.9.	Specific target organ toxicity – repeated exposure	No classification			Conclusive, but not sufficient for classification
3.10.	Aspiration hazard	-			Data lacking
4.1.	Hazardous to the aquatic environment	**Aquatic acute 1**, M = 1, H400: Very toxic to aquatic life. **Aquatic chronic 1**, M = 1, H410: Very toxic to aquatic life with long lasting effects.	1 (acute/chronic)	None	-
5.1.	Hazardous to the ozone layer	None			No classification

[1)] Including specific concentration limits (SCLs) and M-factors
[2)] Data lacking, inconclusive, or conclusive but not sufficient for classification

Labelling:

Pictograms: GHS07, GHS09

Signal word: Warning

Hazard statements: H302: Harmful if swallowed
H410: Very toxic to aquatic life with long lasting effects.

Precautionary statements: P273, P391 and P501

Proposed notes assigned to an entry:

None

Table 4: Proposed classification according to DSD

Hazardous property	Proposed classification	Proposed SCLs	Current classification [1)]	Reason for no classification [2)]
Explosiveness	None			No sign of explosion during thermal and friction tests.
Oxidising properties	None			No exothermic reaction observed.
Flammability	None			Flame immediately extinguished after removal of the heat source.
Other physico-chemical properties	None			Not evaluated
Thermal stability	None			Not evaluated
Acute toxicity	Xn; R22		None	-
Acute toxicity – irreversible damage after single exposure	No classification	-	-	Conclusive, but not sufficient for classification
Repeated dose toxicity	No classification			Conclusive, but not sufficient for classification
Irritation / Corrosion	No classification			Conclusive, but not sufficient for classification
Sensitisation	No classification			Conclusive, but not sufficient for classification
Carcinogenicity	No classification			Conclusive, but not sufficient for classification
Mutagenicity – Genetic toxicity	No classification	-	-	Conclusive, but not sufficient for classification
Toxicity to reproduction – fertility	No classification	-	-	Conclusive, but not sufficient for classification
Toxicity to reproduction – development	No classification	-	-	Conclusive, but not sufficient for classification
Toxicity to reproduction – breastfed babies. Effects on or via lactation	No classification	-	-	Conclusive, but not sufficient for classification
Environment	The active substance fulfils the criteria for classification as N, R 50/53	Concentration classification C ≥25% N: R50/53; 2.5% ≤C < 25% N: R51/53; 0.25% ≤C < 2.5%: R52/53	**None**	-

[1)] Including SCLs
[2)] Data lacking, inconclusive, or conclusive but not sufficient for classification

Labelling:
Indication of danger: Xn, Harmful

	N, Dangerous for the environment	
R-phrases:	R22	Harmful if swallowed.
	R 50/53,	Very toxic to aquatic organisms, may cause long term adverse effects in the aquatic environment
S-phrases:	S2	Keep out of reach of children
	S13	Keep away from food, drink and animal feedstuff
	S36/37	Wear suitable protective clothing/glove
	S46	If swallowed, seek medical advice immediately and show this container or label
	S60	This material and its container must be disposed of as hazardous waste
	S61	Avoid release to the environment. Refer to special instructions/safety data sheets

2 BACKGROUND TO THE CLH PROPOSAL

2.1 History of the previous classification and labelling

Sulfoxaflor is a new active substance developed as an insecticide. There is no previous classification and labelling.

2.2 Short summary of the scientific justification for the CLH proposal

Human Health CLH proposal justification:

R22/H302 is proposed based on the LD_{50} estimated in male rats (1405 mg/kg bw) and female rats (1000 mg/kg bw) and male mice (750 mg/kg bw).

Environment CLH proposal justification:

H400 follows from the lowest acute toxicity value of the active substance for the most sensitive tested aquatic organism with LC_{50} < 1 mg a.s./L (*Chironomus dilutus:* LC_{50} = 0.0.622 mg a.s./L, Gerke, 2008d). A M-factor of 1 is applicable based on 0.1 < LC_{50} ≤1 mg a.s./l.

H410 follows from the lowest chronic toxicity value of the active substance for the most sensitive tested aquatic organism with NOEC ≤ 1 mg a.s./L (*Chironomus riparius*: NOEC = 0.0384 mg/L, Gerke, 2009) and the fact that the active substance is not readily biodegradable and not rapidly biodegradable. A M-factor of 1 is applicable based on 0.01 < NOEC ≤ 0.1 mg/l.

R50 follows from the lowest acute toxicity value of the active substance forthe most sensitive tested aquatic organism with LC_{50} < 1 mg a.s./L (*Chironomus dilutus*: LC_{50} = 0.622 mg a.s./L, Gerke, 2008d;).

R53 follows from the fact that the active substance is not readily biodegradable.

2.3 Current harmonised classification and labelling

Sulfoxaflor is a new active substance. There is no current harmonised classification and labelling.

2.3.1 Current classification and labelling in Annex VI, Table 3.1 in the CLP Regulation

Sulfoxaflor is a new active substance. There is no current harmonised classification and labelling in Annex VI, Table 3.1 in the CLP Regulation.

2.3.2 Current classification and labelling in Annex VI, Table 3.2 in the CLP Regulation

Sulfoxaflor is a new active substance. There is no current harmonised classification and labelling in Annex VI, Table 3.1 in the CLP Regulation.

2.4 Current self-classification and labelling

2.4.1 Current self-classification and labelling based on the CLP Regulation criteria

Not applicable.

3 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

Sulfoxaflor is a new pesticide active substance currently under review for approval to Regulation (EC) No 1107/2009 of the European Parliament and of the Council. The classification and labelling proposal includes mammalian and environmental toxicity endpoints and needs to be evaluated under the CLP Regulation.

PART B

SCIENTIFIC EVALUATION OF THE DATA

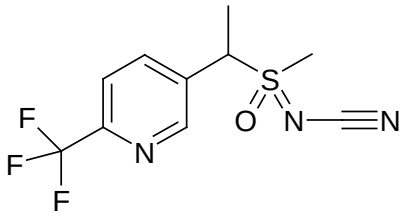
1 IDENTITY OF THE SUBSTANCE

1.1 Name and other identifiers of the substance

Table 5: Substance identity

EC number:	Not available
EC name:	Not available
CAS number (EC inventory):	Not available
CAS number:	946578-00-3
CAS name:	Cyanimide, N-[methoxido[1-[6-(trifluoromethyl)-3-pyridinyl]ethyl]-λ^4-sulfanylidene]-
IUPAC name:	[methyl(oxo){1-[6-(trifluoromethyl)-3-pyridyl]ethyl}-λ6-sulfanylidene]cyanamide
CLP Annex VI Index number:	New active substance - No current entry
Molecular formula:	$C_{10}H_{10}F_3N_3OS$
Molecular weight range:	277.3 g/mol

Structural formula:



1.2 Composition of the substance

Table 6: Constituents (non-confidential information)

Constituent	Typical concentration	Concentration range	Remarks
Sulfoxaflor		≥ 950 g/kg	
Impurities	There are no impurities of toxicological or environmental concern in Sulfoxaflor technical. Sulfoxaflor is a mixture of two diastereomers where the approximate ratio of diastereoisomers 1 and 2 is typically in the range of 40:60 to 60:40, but can vary due to epimerization. The epimerization can occur rapidly depending on pH conditions and other factors. Each diastereomer is composed of two enantiomers (racemic). See IUCLID section 1.4		

Current Annex VI entry: New active substance - No current entry

Table 7: Impurities (non-confidential information)

Impurity	Typical concentration	Concentration range	Remarks
Confidential data	There are no impurities of toxicological or environmental concern in Sulfoxaflor technical. See IUCLID section 1.4		

Current Annex VI entry: Not applicable

Table 8: Additives (non-confidential information)

There are no additives to Sulfoxaflor technical

Additive	Function	Typical concentration	Concentration range	Remarks
None	-	-	-	-

Current Annex VI entry: Not applicable

1.2.1 Composition of test material

Not applicable.

1.3 Physico-chemical properties

Table 9: Summary of physico - chemical properties

Property	Value	Reference	Comment (e.g. measured or estimated)
State of the substance at 20°C and 101,3 kPa	White powder with a sharp odour	Madsen, 2009a FAPC-G-09-15	Observed
Melting/freezing point	112.9°C	Madsen, 2009a FAPC-G-09-15	Measured
Boiling point	No boiling before decomposition point (167.7°C)	Madsen, 2009a FAPC-G-09-15	Measured
Relative density	1.5378	Sarff, 2008 NAFST-08-024	Measured
Vapour pressure	≤ 1.4 µPa at 20°C for pure substance	Turner, 2009 NAFST-08-72	Measured
Surface tension	57.5 mN/m at 20°C for a saturated solution at 90%	Turner, 2009e NAFST-08-75	Measured
Water solubility	568 mg/L at pH7 and 20°C	Turner, 2009b NAFST-08-73	Measured
Partition coefficient n-octanol/water	Pow: 6.34 at pH 7 and 20°C	Turner, 2009d NAFST-08-74	Measured
Flash point	/		
Flammability	Substance burns with a yellow flame which extinguished immediately after removal of the heat source.	Turner, 2009e NAFST-08-75	Measured
Explosive properties	Thermal and friction tests negative	Turner, 2009f NAFST-09-93	Measured
Self-ignition temperature	No auto flammability before melting point at 110°C	Turner, 2009e NAFST-08-75	Measured
Oxidising properties	No temperature change greater than 5°C on the thermogram	Madsen, 2009b	Measured
Granulometry	Not tested		
Stability in organic solvents and identity of relevant degradation products	Not tested		
Dissociation constant	No dissociation between pH 2 and 10	Cathie, 2010 10-003-G	Measured
Viscosity	Not tested		

2 MANUFACTURE AND USES

2.1 Manufacture

This information is provided in PPP DAR Volume 4 Annex C (confidential section).

2.2 Identified uses

This substance is proposed to be used as an insecticide.

3 CLASSIFICATION FOR PHYSICO-CHEMICAL PROPERTIES

3.1 Explosive properties

3.1.1 Summary and discussion of explosive properties for Sulfoxaflor

Based on procedures ASTM E967-92 and ASTM E 537-86, no functional groups associated with Sulfoxaflor are known to have explosive potential. XDE-208 is therefore predicted to be non explosive. The thermal test results showed a yellow flame and the tubes were recovered intact. The friction test results showed no sign of ignition or explosion but slight decomposition indicated by dark mark on porcelain plate.

The thermal and friction tests were negative, therefore Sulfoxaflor is not explosive.

3.1.2 Comparision with criteria

Not applicable.

3.1.3 Conclusions on classification and labelling

Sulfoxaflor is not explosive. No classification required.

3.2 Oxidising properties

3.2.1 Summary and discussion of oxidising properties for Sulfoxaflor

There was no temperature change greater thatn 5^{o}C on the thermogram, therefore Sulfoxalfor has no oxidising properties.

3.2.2 Comparision with criteria

Not applicable.

3.2.3 Conclusions on classification and labelling

Sulfoxaflor is not oxidising. No classification required.

3.3 Flammability properties

3.3.1 Summary and discussion of flammability properties for Sulfoxaflor

Sulfoxaflor was not highly flammable. The technical grade active ingredient (TGAI) burned with a yellow flame, which extinguished immediately after removal of the heat source and did not propagate along the test pile.

3.3.2 Comparision with criteria

Not applicable.

3.3.3 Conclusions on classification and labelling

Sulfoxaflor is not flammable. No classification required.

3.4 Auto-flammability properties

3.4.1 Summary and discussion of auto-flammability properties for Sulfoxaflor

No auto flammability was observed before melting point at 110^{o}C.

3.4.2 Comparision with criteria

Not applicable.

3.4.3 Conclusions on classification and labelling

Sulfoxaflor is not auto-flammable. No classification required.

3.5 Overall conclusion on the classification for physical and chemical properties

Sulfoxaflor is not explosive, oxidising, flammable or auto-flammable and does not classify from a physical and chemical point of view. Therefore, no classification is required.

4 HUMAN HEALTH HAZARD ASSESSMENT

The data presented in this section is reproduced directly from the **Plant Protection Product Draft Assessment Report (DAR) for Sulfoxaflor** either in summary form or as robust study summaries, as appropriate. The Draft Assessment Report (DAR) for Sulfoxaflor is prepared in accordance with Reg. (EC) No. 1107/2009 concerning the placing of Plant Protection Products on the market.

In addition, to relevant CLH report numbering DAR reference numbers are also given for each endpoint for ease of reference. In the case of endpoints that are relevant for hazard identification according to CLP and DSD criteria the text is reproduced directly from the Draft Assessment Report (DAR) for Sulfoxaflor. In this case the study will be headed Study X (Sulfoxaflor DAR, relevant hazard section, DAR number B.6.X etc). The details in brackets will indicate the original location of the data in the DAR. It is also necessary to point out that the figures and tables will be adapted to indicate the CLH report and DAR dual numbering. The in-text citations will remain as they were for the DAR and will not be adapted to match with the CLH report.

4.1 Toxicokinetics (absorption, metabolism, distribution and elimination)

4.1.1 Non-human information

Table 10: Summary table of relevant toxicokinetics studies

Method	Results	Test species/test material	Reference (DAR)
OECD, Guideline 417 (1984), EC, Guideline B.36 (1986) USEPA OPPTS 870.7485; (1998). GLP compliant.	Orally administered Sulfoxaflor was rapidly and extensively absorbed in rats. It was widely distributed in tissues with little metabolism and the majority is rapidly eliminated within 24 hr. Sulfoxaflor is predominantly eliminated in the urine as unchanged parent compound. There was no evidence for bioaccumulation of Sulfoxaflor or its metabolites in tissues.	Rats (F344/DuCrl). Sulfoxaflor, tech/XDE-208 : Lot E2162-34/95.6% (w/w); as two diastereomers in 48.4 / 47.4% ratio	Hansen et al, (2009). DAR B.6.1.3

In the rat, orally administered Sulfoxaflor was extensively and rapidly absorbed from the GI tract without any apparent lag time based on plasma Cmax occurring at 0.5 and 1 hour in female and male rats, respectively, at the low dose of 5 mg/kg and within 2 hours at the high dose of 100 mg/kg. Absorption remained unsaturated at the high dose and dose proportional increases in exposure were observed between the 5 and 100 mg/kg doses. Oral absorption was > 95% in the rat and≥ 87% in mice without any sex differences. The oral bioavailability of Sulfoxaflor, calculated using oral and *iv* AUCs, was at least 94% for both male and female rats.

Sulfoxaflor was well distributed with detectable levels of radioactivity found in all tissues at C_{max} (0.5-2 hours after dosing) and ½C_{max} (6-8 hours after dosing) with the highest radioactivity associated with sites of entry and excretion (GI tract, liver, kidney, and urinary bladder). Plasma and tissues were typically cleared of Sulfoxaflor within 48 hours; 57-79, 86-99 and 93-100 percent of the dose was eliminated within 12, 24 and 48 hours of dosing, respectively. Consequently, only a total of 0.2-1.3% of the administered dose remained in the tissues of rats 7 days after dosing.

Sulfoxaflor was poorly metabolised. More than 93% of the eliminated radioactivity in urine and faeces was parent Sulfoxaflor. Six additional radiolabelled compounds were detected in urine (4 compounds) and faeces (2 compounds), but only one exceeded 1.0%, a urinary glucuronide conjugate of the Sulfoxaflor metabolite X11721061, which accounted for 2 – 4% of the administered dose. Structures of the five minor metabolites (< 1% of the dose) were not determined.

Sulfoxaflor was rapidly excreted in urine without any sex difference. Excretion of the absorbed Sulfoxaflor was also not affected by the dose level (5 mg/kg versus 100 mg/kg), or number of doses. The majority of radioactivity was eliminated in urine; 57-74%, 77-90% and 86- 97% was recovered in rat urine at 12, 24 and 48 hours, respectively. The fate of the rat *iv* administered Sulfoxaflor was similar to that after oral dosing in rats and mice. Biliary elimination was low, accounting for only 6 – 9% of the *iv* administered Sulfoxaflor recovered in the faeces. Elimination of Sulfoxaflor from plasma was bi-exponential with most of the elimination occurring during the α-phase with elimination half-life of 4-6 hours, while the half-life of the β-phase was 39-45 hours.

Sulfoxaflor does not bioaccumulate in tissues or plasma. Almost the entire administered dose (93-100%) was eliminated from the body within 48 hours with ≤ 1% of the dose remaining in tissues 7 days after a single oral / iv or repeated (15-daily doses) oral dosing regimen.

4.1.2 Human information

No data available.

4.1.3 Summary and discussion on toxicokinetics

Sulfoxaflor was extensively and rapidly absorbed. It is well distributed with detectable levels of radioactivity found in all tissues at C_{max}. Sulfoxaflor was poorly metabolised. More than 93% of the eliminated radioactivity in urine and faeces was parent Sulfoxaflor. Sulfoxaflor was rapidly excreted in urine without any sex difference and does not bio accumulate in tissues or plasma.

4.2 Acute toxicity

Table 11: Summary table of relevant acute toxicity studies

Method	Results	Remarks	Reference (DAR)
Rat Oral LD_{50} (Up and Down Procedure/ OECD 425)	Males 1405 mg/kg bw Females 1000 mg/kg bw	Strain: F344/DuCrl Sulfoxaflor (95.6 % w/w) Lot E2162-34	Brooks, K.J., et al., (2008). DAR B.6.2.1.1
Mouse oral LD_{50} (Up and Down Procedure/ OECD 425)	750 mg/kg bw	Strain: male Crl:CD1(ICR) Sulfoxaflor (95.6 % w/w) Lot E2162-34	Brooks, K.J., et al. (2008) DAR B.6.2.1.2
Rat dermal LD_{50} (OECD 402)	> 5000 mg/kg	F344/DuCrl Sulfoxaflor (95.6 % w/w) Lot E2162-34	Durando, J. (2008) DAR B.6.2.2.1
Rat Inhalation LC_{50} (OECD 403)	>2.09 mg/l	F344/DuCrl Sulfoxaflor (95.6 % w/w) Lot E2162-34 Highest attainable concentration	Krieger, S.M., & Radtke, B.J., (2009) DAR B.6.2.3.1

4.2.1 Non-human information

4.2.1.1 Acute toxicity: oral

(Sulfoxaflor DAR, Acute Toxicity sections B.6.2.1.1 to B.6.2.3.1)

Study 1 (Rat):

Method	Results	Remarks	Reference (DAR)
Rat Oral LD_{50} (Up and Down Procedure/ OECD 425)	Males 1405 mg/kg bw Females 1000 mg/kg bw	Strain: F344/DuCrl Sulfoxaflor (95.6 % w/w) Lot E2162-34	Brooks, K.J., et al., 2008. DAR B.6.2.1.1

The purpose of this study was to determine the median lethal dose (LD_{50}) of Sulfoxaflor (purity 95.6% w/w) in male and female F344/DuCrl rats. Based on an estimated LD_{50} of 1000 mg/kg, a starting dose level of 630 mg/kg of Sulfoxaflor in 0.5% aqueous methylcellulose was administered to one male and one female rat by oral gavage. Both

animals survived. Following the Up and Down Procedure, five additional males were dosed at levels of 1000 (2 animals), 1580 (1 animal) or 2000 (1 animal) mg/kg and five additional females were dosed at levels of 1000 (3 animals) or 1580 (1 animal) mg/kg. In total, four animals died on test day 1 (1 female at 1000 mg/kg bw, 1 female at 1580 mg/kg bw, 1 male at 1000 mg/kg bw and 1 male at 2000 mg/kg bw). The remainder survived the 14-day observation period.

All animals (2 male/2 female) dosed with 630 mg/kg Sulfoxaflor survived the 14-day observation period and gained body weight during the study. One animal/sex exhibited clinical signs consisting of muscle tremors and decreased activity on test day 1, which resolved by test day 2. All rats dosed with either 1000 mg/kg (2 male/3 female), 1580 mg/kg (1 male/1 female) or 2000 mg/kg (1 male), exhibited muscle tremors, twitches, and/or tonoclonic convulsions. Other clinical signs in some animals included decreased activity, decreased reactivity, decreased faeces, eyelids partially closed, hair standing up, laboured respiration, and various types of soiling, all of which resolved by test day 6. Other ranked observations noted in several of these animals were; increased salivation, increased lacrimation, abnormal gait, inability to walk, increased reactivity to stimuli, decreased resistance to removal, and decreased responsiveness to touch on test day 1.

Time of peak effect was approximately 2 hours post-dosing for most animals. No gross pathological observations were noted for any of the surviving animals at the conclusion of the 14-day observation period. This acute oral toxicity study of Sulfoxaflor was performed in accordance with GLP and OECD/EU guidelines and found acceptable. The following LD_{50} values were established: LD_{50} = 1405 mg/kg bw (male fasted): 1000 mg/kg bw (female fasted).

Study 2 (Mice):

Method	Results	Remarks	Reference (DAR)
Mouse oral LD_{50} (Up and Down Procedure/ OECD 425)	750 mg/kg bw	Strain: male Crl:CD1(ICR) Sulfoxaflor (95.6 % w/w) Lot E2162-34	Brookes, K .J., *et al.* (2008) B.6.2.1.2

The purpose of this study was to determine the median lethal dose (LD_{50}) of Sulfoxaflor (purity 95.6% w/w) in male Crl:CD1(ICR) mice. Based on an estimated LD_{50} of 1000 mg/kg, a starting dose level of 750 mg/kg of Sulfoxaflor in 0.5% aqueous methylcellulose was administered to one male mouse by oral gavage. This animal died approximately 1 hour post dosing. Following the Up and Down Procedure, four additional males were dosed at levels of 560 (1 animal), 750 (2 animals) or 1000 (1 animal) mg/kg bw. In total, two animals died on test day 1 (1 at 750 mg/kg bw and 1 at 1000 mg/kg bw approximately 4 hours post dosing). The remainder survived the 14-day observation period.

The one animal dosed at 560 mg/kg bw survived and gained body weight throughout the study period. Clinical signs consisted of laboured respiration, muscle convulsions, decreased activity, and decreased resistance to removal on test day 1, which resolved by test day 2. No gross internal findings were observed at necropsy.

Of the three animals dosed at 750 mg/kg bw, one animal died approximately 1 hour post-dosing. Clinical signs noted prior to death included muscle convulsions and increased activity. The surviving two animals exhibited decreased activity, muscle twitches, tremors, and/or convulsions, decreased responsiveness to touch or increased reactivity to stimuli on

test day 1, which resolved by test day 2. The two surviving animals lost body weight by test day 2, and then gained weight throughout the remainder of the study period. No gross internal findings were observed at necropsy.

The one animal dosed at 1000 mg/kg bw died within 4 hours post-dosing. Clinical signs noted prior to death included muscle twitches, tremors, and convulsions, increased reactivity to stimuli, and increased responsiveness to touch. No gross internal findings were observed at necropsy.

The estimated acute oral LD_{50} of Sulfoxaflor in male Crl:CD1(ICR) mice is 750 mg/kg body weight.

4.2.1.2 Acute toxicity: dermal

Method	Results	Remarks	Reference (DAR)
Rat dermal LD_{50} (OECD 402)	> 5000 mg/kg	F344/DuCrl Sulfoxaflor (95.6 % w/w) Lot E2162-34	Durando, J. (2008) B.6.2.2.1

The purpose of this study was to determine the potential for Sulfoxaflor (purity 95.6% w/w) to produce toxicity from a single topical application to both male and female F344/DuCrl rats. Under the conditions of this study, the single dose acute dermal LD_{50} of the test substance is > 5,000 mg/kg per body weight in both sexes.

5000 mg of the test substance per kilogram of body weight was moistened with distilled water and then applied to the skin of ten healthy rats for 24 hours. The animals were observed for mortality, signs of gross toxicity, and behavioural changes at least once daily for 14 days. Body weights were recorded prior to application and again on Days 7 and 14 (termination). Necropsies were performed on all animals at terminal sacrifice.

All animals survived, gained body weight, and appeared active and healthy during the study. There were no signs of gross toxicity, dermal irritation, adverse pharmacological effects, or abnormal behaviour. No gross abnormalities were noted for any of the animals when necropsied at the conclusion of the 14-day observation period. The dermal toxicity of Sulfoxaflor is low.

4.2.1.3 Acute toxicity: inhalation

Method	Results	Remarks	Reference (DAR)
Rat Inhalation LC_{50} (OECD 403)	>2.09 mg/l	F344/DuCrl Sulfoxaflor (95.6 % w/w) Lot E2162-34 Highest attainable concentration	Krieger, S.M., & Radtke, B. J., (2009) DAR B.6.2.3.1

The purpose of this study was to determine the acute inhalation toxicological properties of Sulfoxaflor (purity 95.6%). Due to the physical-chemical properties of Sulfoxaflor, there were significant technical problems in producing a stable respirable aerosol at the 5 mg/L limit test during the preliminary generation method development phase of this study. Repeated attempts consistently resulted in a mass mean aerodynamic diameter greater than 4 µm of particulate Sulfoxaflor. Therefore, groups of five rats/sex were exposed for four hours, using a nose-only inhalation exposure system, to a time-weighted average (TWA) chamber concentration of 2.09 mg Sulfoxaflor per litre of air. This was the highest attainable

concentration with a particle size distribution mass median aerodynamic diameter (MMAD) of 1-4 µm. Animals were then observed for 15 days.

All animals survived the 4-hour exposure as well as the two week post-exposure study period. Mean body weight losses were noted for both male and female animals on test day 2; pre-exposure mean body weight values were exceeded on test day 4. Clinical effects noted during the exposure period were limited to soiling of the haircoat in 2/5 females. Post-exposure, clinical effects were limited to perineal soiling in 2/5 females; all animals appeared normal by test day 4. No gross internal findings were observed at necropsy.

The mean mass aerodynamic diameter (MMAD) averaged 3.6 µm with an average geometric standard deviation (GSD) of 1.33. Approximately 12% of the particle mass was contained in a size fraction with an aerodynamic diameter less than 1.3 µm. Approximately 96% of the particulate mass was present in size fractions with an aerodynamic diameter less than 6.1 µm.

The four-hour LC_{50} of inhaled particulate Sulfoxaflor is > 2.09 mg/L for male and female Fischer 344/DUCRL rats, which was the highest attainable concentration with a particle size distribution mass median aerodynamic diameter (MMAD) of 1-4 µm.

4.2.1.4 Acute toxicity: other routes

No data available.

4.2.2 Human information

No data available.

4.2.3 Summary and discussion of acute toxicity

A number of studies were available for Sulfoxaflor performed in rats and mice.

In the two oral studies, low oral toxicity is observed in rodent species. Time of peak effect was approximately 2 hours post-dosing for most animals. No gross pathological observations were noted for any of the surviving animals at the conclusion of the 14-day observation period. The lowest oral LD_{50} values obtained were 1000 mg/kg bw and 750 mg/kg bw in fasted female F344/DuCrl rats and male Crl:CD1(ICR) mice, respectively.

In the dermal toxicity study low toxicity was seen in the limit test where a dose of 5000 mg/kg bw was administered to both male and female F344/DuCrl rats. All animals survived, gained body weight, and appeared active and healthy during the study. There were no signs of gross toxicity, dermal irritation, adverse pharmacological effects, or abnormal behaviour. No gross abnormalities were noted for any of the animals when necropsied at the conclusion of the 14-day observation period.

In the inhalation study with F344/DuCrl rats, no deaths occurred and the LC_{50} >2.09 mg/L for male and female rats was determined by nose-only exposure under dynamic conditions. All animals survived the four-hour exposure to the test material as well as the two-week post-exposure period.

4.2.4 Comparison with CLP and DSD classification criteria

The lowest LD_{50} values of Sulfoxaflor were 1000 mg/kg bw (female rat) and 750 mg/kg bw

(male mice) via the oral route. Sulfoxaflor is not considered acutely toxic via dermal and inhalation routes.

CLP

According to the CLP Regulation (EC) No. 1272/2008, Sulfoxaflor should be classified as Acute Tox. Cat. 4 with the hazard statement H302 "Harmful if swallowed", because the LD_{50} is within the limits, 300 < ATE ≤ 2000 (oral, mg/kg bw).

67/548/EEC

The classification according to 67/548/EEC is Xn; R22 "harmful if swallowed", because the LD_{50} is within the limits, 200 < LD_{50} ≤ 2000 mg/kg.

4.2.5 Conclusions on classification and labelling

	CLP Regulation	**Directive 67/548/EEC (DSD)**
Resulting harmonised classification (Annex VI, CLP Regulation)	Acute Tox. 4 (H302)	Xn: R22

4.3 Specific target organ toxicity – single exposure (STOT SE)

The results from the standard acute and acute neurotoxicity studies submitted in support of sulfoxaflor registration do not indicate that there is specific organ toxicity following a single exposure. The effects observed in the standard acute toxicity studies were generalised and systemic in nature, occurred at high doses of sulfoxaflor, involved small numbers of animals, were transitory in nature without significant functional change in any organ system and are not considered to support STOT SE classification.

4.3.1 Summary and discussion of Specific target organ toxicity – single exposure

4.3.1.1 Summary of Standard Acute Studies

In the rat oral acute study (section 4.2.1.1, study 1), all rats dosed with either 1000 mg/kg (2 male/3 female), 1580 mg/kg (1 male/1 female) or 2000 mg/kg (1 male), exhibited muscle tremors, twitches, and/or tonoclonic convulsions. Other clinical signs in some animals included decreased activity, decreased reactivity, decreased faeces, eyelids partially closed, hair standing up, laboured respiration, and various types of soiling, all of which resolved by test day 6. Other ranked observations noted in several of these animals were; increased salivation, increased lacrimation, abnormal gait, inability to walk, increased reactivity to stimuli, decreased resistance to removal, and decreased responsiveness to touch on test day 1.

In the mouse oral acute study (section 4.2.1.1, study 2), clinical signs in the animal dosed at 560 mg/kg bw consisted of laboured respiration, muscle convulsions, decreased activity, and decreased resistance to removal on test day 1, which resolved by test day 2. Surviving animals dosed at 750 mg/kg bw exhibited decreased activity, muscle twitches, tremors, and/or convulsions, decreased responsiveness to touch or increased reactivity to stimuli on test day 1, which resolved by test day 2.

In the rat acute dermal study (section 4.2.1.2), there were no signs of gross toxicity, dermal irritation, adverse pharmacological effects, or abnormal behaviour.

In the rat acute inhalation study (section 4.2.1.3), clinical effects noted during the four-hour exposure period were limited to soiling of the hair coat in two female rats. Post-exposure, clinical effects were limited to perineal soiling in 2/5 females.

4.3.1.2 Summary and discussion of Acute Neurotoxicity Study

4.3.1.2-1 Summary table of acute neurotoxicity study

Rat studies				
Test system/species/dose levels	**Significant Findings**	**NOAEL**	**LOAEL**	**Reference/ DAR reference**
Rat single oral gavage dose (F344/DuCrl) OECD 424: 0, 7.5, 75, or 750 mg sulfoxaflor /kg body	-decreased or absent faeces -red perioral soiling -perineal urine soiling (females only) (750 mg/kg bw/day). -Decreased motor activity on day 1 (75 mg/kg bw/day)	25 mg/kg bw/day	75 mg/kg bw/day	Marty, M. S. 2010 B.6.7.1/1

In an acute neurotoxicity study (DAR B.6.7.1) ten male and ten female F344/DuCrl rats per group were given a single, oral gavage dose of 0, 7.5, 75, or 750 mg sulfoxaflor/kg body weight to evaluate the potential for acute neurotoxicity. Body weights were recorded and a functional observational battery (FOB) and test for motor activity were conducted pre-exposure (baseline), the day of dosing (day 1, time-of-peak effect), day 8, and day 15. The FOB included hand-held and open-field observations as well as measurements of grip performance, landing foot splay and rectal temperature. Clinical observations were conducted on days 2, 3, and 4. At the end of the study all rats from the control and high-dose group and five rats/sex/low- and mid-dose group were perfused for histopathological evaluation of the central and peripheral nervous systems, which was conducted on all of the control and high-dose group rats. A second motor activity study at dose levels of 0, 2.5, 7.5 and 25 mg/kg was conducted to investigate whether an apparent decrease in motor activity at 7.5 mg/kg was reproducible or treatment-related, and to establish a clear no-observed-adverse-effect level (NOAEL). Motor activity was the only endpoint examined in this study phase.

One female rat given 750 mg/kg died following dosing on day 1, but the cause of death could not be determined. Treatment-related categorical observations on day 1 in males and females given 750 mg/kg included increased incidences of muscle tremors and twitches, convulsions, splayed hindlimbs and perineal urine soiling. Treatment-related ranked FOB observations on day 1 in males and females given 750 mg/kg were as follows: increased lacrimation and salivation, decreased pupil size and response to touch, increased level of urination (females only), decreased level of open-field activity and gait abnormalities. There were no treatment-related ranked or categorical FOB observations present on day 8 or day 15 in males or females given 750 mg/kg. There were no treatment-related ranked or categorical observations in males or females given 7.5 or 75 mg/kg during any FOB.

There was a treatment-related decrease in body weight of the 750 mg/kg group when compared to controls on days 8 and 15, which was more prominent in males than in females. There was a treatment-related decrease in rectal temperature of the 750 mg/kg group when

compared to controls on day 1, which was not present in the subsequent examinations on days 8 and 15. There were no treatment-related effects in grip performance or landing foot splay.

There was a treatment-related decrease in the day 1 total motor activity and an effect on the distribution of motor activity counts of males and females given 75 or 750 mg/kg. The effect on total motor activity of animals given 7.5 mg/kg was considered equivocal on day 1. There were no effects on motor activity on days 8 or 15 in rats of any dose group. In the follow-up motor activity study, there were no treatment-related effects on total motor activity or on the distribution of motor activity counts for males and females given 2.5, 7.5 or 25 mg/kg when compared to controls.

Treatment-related clinical findings on days 2, 3 or 4 were limited to males and females given 750 mg/kg, and included decreased or absent faeces, red perioral soiling, and perineal urine soiling (females only). There were no treatment-related gross or histopathological findings in the central or peripheral nervous system.

The lowest-observed-adverse-effect-level (LOAEL) for neurotoxicity was 75 mg/kg based on decreased motor activity observed on day 1. The no-observed-adverse-effect-level (NOAEL) was 25 mg/kg. No treatment-related effects were observed for neuropathology; therefore the NOAEL for neuropathology was ≥ 750 mg/kg, the highest dose level tested.

4.3.2 Comparison with CLP and DSD classification criteria

Classification with regard to STOT SE is based on the observance of significant, non-lethal target organ/systemic toxic effects arising from a single exposure, relevant to human health that can impair function, both reversible and irreversible, immediate and/or delayed. It is determined by expert judgement and on the basis of the weight of all evidence available. The nature and severity of the effect relative to EU guidance values determine the category into which a substance may be placed.

Significant functional changes were not observed in any of the acute studies mentioned above. Clinical signs when present were transitory in nature and mainly affected motor function. The acute neurotoxicity study showed there were no treatment-related gross or histopathological findings in the central or peripheral nervous system.

4.3.3 Conclusions on classification and labelling

No recommendation for STOT SE.

4.4 Irritation

4.4.1 Skin irritation

(Sulfoxaflor DAR, Acute Toxicity section B.6.2.4.1)

Table 12: Summary table of relevant skin irritation studies

Method	Results	Remarks	Reference
Skin Irritation (rabbit): OECD 404 (2002)	Individual mean score for 24,48 and 72 hrs respectively: - Erythema: 0.33, 0.67, 0.33 - Oedema: 0, 0, 0.33	New Zealand albino, all female.	Durando, J. (2008). DAR B.6.2.4.1

4.4.1.1 Non-human information

In a primary dermal irritation study, three female young adult New Zealand albino rabbits were dermally exposed to 0.5 g of Sulfoxaflor, moistened with distilled water, for 4 hours to one 6 cm^2 intact dose site on each animal. Animals were then observed at 1, 24, 48 and 72 hours post-patch removal. Irritation was scored by the method of Draize.

Within one-hour post-patch removal, all 3/3 treated sites exhibited very slight erythema (grade 1 on the Draize scale, barely perceptible) and very slight oedema (grade 1 on the Draize scale, barely perceptible). The overall incidence and severity of irritation decreased thereafter. All animals were free of dermal irritation within 72-hours.

Under the conditions of this study, Sulfoxaflor is not classified as a dermal irritant.

4.4.1.2 Human information

No data available.

4.4.1.3 Summary and discussion of skin irritation

Dermally applied Sulfoxaflor was found to be very slightly irritating with complete resolution by 72 hours post exposure.

4.4.1.4 Comparison with CLP and DSD classification criteria

Dermal scores indicated a very slight irritant response but these scores were well below EU trigger values under CLP and DSD classification systems. Parameter scores did not exceed 1 for any animaland were completely resolved within 72 hours post-patch removal.

<u>CLP</u>

Classification not required, EU trigger values were not exceeded. Under CLP, the major criterion for the irritant category is that at least 2 of 3 tested animals have a mean score of $2.3 \leq 4.0$

<u>67/548/EEC</u>

Classification not required, EU trigger values were not exceeded. Under DSD, the major criterion to classify for skin irritation is that the mean value of the scores for either erythema and eschar formation or oedema formation, is ≥ 2 for each animal and is observed in two or more animals in a three animal study.

4.4.1.5 Conclusions on classification and labelling

Classification is not required as EU trigger values were not exceeded according to CLP or DSD classification systems.

4.4.2 Eye irritation

(Sulfoxaflor DAR, Acute Toxicity section B.6.2.5.1)

Table 13: Summary table of relevant eye irritation studies

Method	Results	Remarks	Reference
Eye Irritation (rabbit): OECD 405 (2002)	Individual animal mean score for 24,48 and 72 hrs respectively: - Cornea: 0, 0, 0 - Iritis: 0, 0.33, 0 - Conjunctiva (redness): 0.33, 1, 1 - Conjunctiva (chemosis): 0, 0.33, 0	New Zealand albino, all male.	Durando, J. (2008) DAR B.6.2.5.1

4.4.2.1 Non-human information

The purpose of this study was to determine the eye irritation potential of Sulfoxaflor. In a primary eye irritation study, 0.1 mL (0.05 g) of Sulfoxaflor was instilled into the conjunctival sac of the right eye in three male young adult New Zealand White albino rabbits. Animals were then observed at 1, 24, 48 and 72 hours post-instillation. Irritation was scored by the method of Draize.

There was no corneal opacity observed in any treated eye during the study. One hour after test substance instillation, all 3/3 treated eyes exhibited positive conjunctivitis and iritis was evident in 2/3 eyes. The overall incidence and severity of irritation decreased with time. All animals were free of ocular irritation within 72 hours.

Under the conditions of this study, Sulfoxaflor does not meet the criteria for classification as an eye irritant.

4.4.2.2 Human information

No data available.

4.4.2.3 Summary and discussion of eye irritation

All animals appeared active and healthy during the study. Apart from very slight eye irritation, there were no signs of gross toxicity, adverse pharmacological effects, or abnormal behaviour. All animals gained weight throughout the study.

4.4.2.4 Comparison with CLP and DSD classification criteria

Eye irritation scores indicated a very slight irritant response but these scores were well below EU trigger values under CLP and DSD classification systems.

<u>CLP</u>

Classification is not required, as EU trigger values under CLP were not exceeded in any

animal (a positive response in at least 2 of 3 tested animals was not observed; the criteria for a positive response in a single animal are mean gradings for conjunctiva – redness (or chemosis): ≥ 2.0; iritis: ≥ 1; corneal opacity: ≥ 1).

67/548/EEC

Classification is not required, as EU trigger values under DSD were not exceeded in any animal (a positive response in at least 2 of 3 tested animals was not observed; the criteria for a positive response in a single animal are mean gradings for conjunctiva – redness: ≥ 2.5; conjunctiva – chemosis: ≥ 2.0; iritis: ≥ 1 < 1.5; corneal opacity: ≥ 2 < 3).

4.4.2.5 Conclusions on classification and labelling

Classification is not required as EU trigger values according to CLP or DSD classification systems were not exceeded for any animal.

4.4.3 Respiratory tract irritation

There is no data to indicate evidence of respiratory tract irritation. The acute rat inhalation study provides no evidence for impairment of the respiratory system. Both the rabbit acute dermal irritation study and rabbit acute eye irritation study indicate a lack of irritant potential on the dermis and mucous membranes respectively.

4.4.3.1 Non-human information

In the rat acute inhalation study (section 4.2.1.3), clinical effects noted during the four-hour exposure period were limited to soiling of the hair coat in two female rats. Post-exposure, clinical effects were limited to perineal soiling in 2/5 females. There were no observations recorded with respect to breathing and chest sounds that might have indicated an underlying inflammation of the respiratory tract. Additionally, there were no gross internal findings observed at necropsy.

In the rabbit acute dermal irritation study (section 4.4.1.1) with exposure to 0.5 g of Sulfoxaflor, moistened with distilled water, for 4 hours to one 6 cm^2 intact dose site per animal, very slight erythema and oedema were initially noted and fully resolved by 72 hours. The grading of the response is described as “barely perceptible” and does not indicate any concern for erosion or irritation of dermal surfaces. There were no other signs of gross toxicity, adverse pharmacologic effects, or abnormal behavior. There were no observations recorded with respect to breathing and chest sounds.

In the rabbit eye irritation study (section 4.4.2.1), minor conjunctivitis and iritis responses were observed. All responses were completely resolved within 72 hours. There was no corneal opacity at any stage. There were no observations to support the concern that sulfoxaflor should be classed as an irritant to the eye and by extension as an irritant to mucous membranes that might have indicated an underlying potential for inflammation of the respiratory tract.

28 day and 90 day rodent inhalation studies were not conducted. Sulfoxaflor applied dermally in a rat 28 day repeat dose dermal toxicity study did not reveal any sign of dermal irritation at any dose level up to 1000mg/kg bw/day (section 4.7.1.3). .

4.4.3.2 Human information

No data available.

4.4.3.3 Summary and discussion of respiratory tract irritation

There is no direct evidence for respiratory tract irritation. There is no evidence of respiratory tract involvement during the rat acute inhalation study. There is no indirect evidence form acute dermal irritation and eye irritation studies. There is no inference of respiratory involvement from the 28-day rat repeat dose dermal toxicity study.

4.4.3.4 Comparison with CLP and DSD classification criteria

Not relevant in this particular context.

4.4.3.5 Conclusions on classification and labelling

No recommendation for classification with respect to respiratory tract irritation.

4.5 Corrosivity

Table 14: Summary table of relevant corrosivity studies

Method	Results	Remarks	Reference
Not applicable	Not applicable	Not applicable	Not applicable

4.5.1 Non-human information

No evidence of corrosivity from any of the dermal contact studies. There is no evidence of corrosivity from the rat acute dermal toxicity study (section 4.2.1.2) or the 28-day rat repeat dose dermal toxicity study (section 4.7.1.3). Similarly, there is no evidence of surface damage or corrosivity from more appropriate studies such as the acute rabbit dermal irritation and rabbit eye irritation studies.

4.5.2 Human information

No data.

4.5.3 Summary and discussion of corrosivity

There is no evidence of corrosivity.

4.5.4 Comparison with CLP and DSD classification criteria

Not required.

4.5.5 Conclusions on classification and labelling

Not applicable.

4.6 Sensitisation

4.6.1 Skin sensitisation

(Sulfoxaflor DAR, Acute Toxicity section B.6.2.6.1)

Table 15: Summary table of relevant skin sensitisation studies

Method	Results	Remarks	Reference
Local Lymph Node assay (mouse): OECD 429 (2002)	Mice treated with 5%, 25% and 50% Sulfoxaflor displayed a proliferative response with Stimulation Indices (SI) that were 1.0, 1.1, and 1.0, respectively, in comparison to vehicle-treated mice.	Female CBA/J mice.	Wiescinski, C.M. and Sosinski, L.K. (2008)

4.6.1.1 Non-human information

The Local Lymph Node Assay (LLNA) was conducted to assess the potential of Sulfoxaflor Technical Grade Active Ingredient (TGAI) to cause contact sensitization by measuring lymphocyte proliferative responses from auricular lymph nodes following topical application of the test material to the mouse ear. Six female mice/group received 5%, 25%, or 50% of Sulfoxaflor or vehicle (DMSO) or 30% α-hexylcinnamaldehyde (HCA; positive control) on days 1-3. On day 6, uptake of 3H-thymidine into the auricular lymph nodes draining the site of chemical application was measured five hours post administration.

During the screening study, mice were treated with three daily applications of 1%, 5%, 10%, 20%, 40%, or 50% Sulfoxaflor technical grade. Erythema was absent and body weights were unaffected at all dose levels.

Based on the results of the screen, 50% Sulfoxaflor was tested in the LLNA along with 25% and 5% to characterise the dose response. Erythema was absent and body weights were unaffected in all dose groups. There were no treatment-related mortalities. Mice treated with 5%, 25% and 50% Sulfoxaflor displayed a proliferative response with Stimulation Indices (SI) that were 1.0, 1.1, and 1.0, respectively, in comparison to vehicle-treated mice.

Proper conduct of the LLNA was demonstrated via the positive and unequivocal response from the positive control, 30% HCA, which elicited a stimulation index (SI) that was 12.0 in comparison to vehicle-treated mice, thus confirming the validity of the protocol used for this study.

Sulfoxaflor is not a dermal sensitiser under the conditions of this study. No classification is necessary. .

4.6.1.2 Human information

No data available.

4.6.1.3 Summary and discussion of skin sensitisation

Mice treated with 5%, 25% and 50% Sulfoxaflor displayed no proliferative response with Stimulation Indices (SI) that were 1.0, 1.1, and 1.0, respectively, in comparison to vehicle-

treated mice. Thus, there is no evidence in this study to suggest sulfoxaflor has sensitising potential. These values were well below all EU classification criteria which stipulate that a significant skin sensitizing effect only exists where the stimulation index is greater than or equal to 3.

4.6.1.4 Comparison with CLP and DSD classification criteria

In this study, Sulfoxaflor is not a dermal sensitiser according to the criteria laid out for both CLP and DSD. No classification is necessary. In the positive control, 30% HCA caused skin sensitisation (SI = 12.0), confirming the validity of the protocol used for this study.

CLP

Classification is not warranted because there is no evidence of sensitisation. The trigger value that determines whether a significant skin sensitising effect exists and if classification is warranted (SI ≥ 3) was not exceeded. Furthermore, because the SI value is close to unity no further refinement or comparison with the criteria is required (as set out in the 2nd ATP to the CLP Regulation (EU 286/2011) with respect to sensitisation and classification into subcategories 1A or 1B).

67/548/EEC

There is no evidence for sensitisation. Classification is not required as the trigger value for classification under DSD (SI ≥ 3) was not exceeded.

4.6.1.5 Conclusions on classification and labelling

Classification is not applicable.

4.6.2 Respiratory sensitisation

No data available.

Table 16: Summary table of relevant respiratory sensitisation studies

Method	**Results**	**Remarks**	**Reference**
Not applicable	Not applicable	Not applicable	Not applicable

4.6.2.1 Non-human information

No data available.

4.6.2.2 Human information

No data available.

4.6.2.3 Summary and discussion of respiratory sensitisation

No data available.

4.6.2.4 Comparison with CLP and DSD classification criteria

No data available.

4.6.2.5 Conclusions on classification and labelling

No data available.

4.7 Repeated dose toxicity

Table 17: Summary table of relevant repeated dose toxicity studies

Rat studies				
Test system/species/dose levels	**Significant Findings**	**NOAEL**	**LOAEL**	**Reference/ DAR reference**
28-d rat (F344/DuCrl) OECD 407: 0, 300, 1000, 2000 ppm, or 3000 ppm *equiv. to* 0, 24.8, 79.4, 155, or 205 mg/kg/day for males and 0, 26.5, 88.3, 170 or 192 mg/kg/day for females	↓↓food consumption with body weight loss (3000 ppm) -altered haematology: ↑haematocrit, reticulocyte counts, platelets. -↑ serum cholesterol and protein, ↓ ALP ↑Liver abs. and rel. wt -Hepatocellular hypertrophy, vacuolisation (fatty change) -Splenic erythroid extramedullary hematopoiesis	300 ppm *equiv to* 24 mg/kg bw/day (males) and 26 mg/kg bw/day (females)	1000 ppm *equiv. to* 79 mg/kg bw/day (males) and 88 mg/kg bw/day (females)	Yano, *et al.*, 2009b B6.3.1/2
90-d rat (F344/DuCrl) OECD 424: 0, 100, 750, 1500 ppm, or 3000 ppm *equiv. to* 0, 6.36, 47.6, or 94.9 mg/kg/day for males and 0, 6.96, 51.6, or 101 mg/kg/day for females	-↓food consumption and weight gain -↑Liver wt -↑cholesterol. -liver histopath (hypertrophy, necrosis and vacuolisation)	100 ppm *equiv. to* 6.36 mg/kg bw/day	750 ppm *equiv. to* 47.6/51mg/kg bw/day	Yano, *et al.*, 2009 B.6.3.2/1
28-d rat dermal (F344/DuCrl) OECD 410: 0, 100, 500 and 1000 mg/kg bw/day semi-occluded	-↑Cholesterol (males) -slight ↑liver wt (slight) -liver hypertrophy (slight)	1000 1000	- Not determined	Thomas, 2009 B.6.3.7
Mouse studies				
<u>28-d mouse</u> (Crl:CD1/(ICR) OECD 407: 0, 300, 1500 and 3500 ppm *equiv to* 0, 43.9/53, 230/273, 524/638 mg/kg bw/day (male/female)	-↑Liver wt -↑ALT, AST, triglyceride, ALP -Hepatocellular hypertrophy, fatty change, some necrosis (males), vacuolisation, mitotic	300 ppm *equiv. to* 53 /43.9 mg/kg bw/day (males/females)	1500 ppm *equiv to* 230/273 mg/kg bw/day (males/females)	Thomas, *et al.*, 2008 B.6.3.1/3b

	figures -↑adrenal weight in males (abs and rel) -hypertrophy of the zona fasiculata of adrenal cortex (males)			
90-d mouse (Crl:CD1/(ICR) OECD 408: males; 0, 100, 750 and 1250 ppm *equiv to* 0, 12.8, 98.0 or 166 mg/kg/day and females; 0, 100, 1500 or 3000 ppm *equiv to* 0, 16.2, 247 or 489 mg/kg/day	-↑Liver wt -↑ALT, AST, ALP, cholesterol, bilirubin -liver histopath (necrosis and fatty change) -↑adrenal weight -adrenal hypertrophy -haematopoiesis (spleen)	100 ppm (13 and 16 mg/kg bw/day)	Males: 750 ppm equiv to 98 mg/kg bw/day. Females: 1500 ppm equiv to 247 mg/kg bw/day	Thomas, *et al.*, 2010 B.6.3.2/2
Dog studies				
28-day Palatability probe/beagle dog/(dietary/capsule/gavage)/500 ppm or 15 mg/kg.bw/day	Oral gavage exposure at 15 mg/kg/day technical grade Sulfoxaflor was tolerable route and concentration for the test material, as determined by adequate food consumption and tolerable in-life clinical signs.	Not applicable	Not applicable	Stewart, (2009). B.6.3.1/4
90-d dog (Beagle) OECD 409: Oral gavage at 0, 1, 3, and 10 (days 1-5)/6 mg/kg bw/day (days 5-90)	↓↓food consumption, weight loss and reduced weight gain ↑Liver wt	10	6.0	Stewart, 2010 (081054). B.6.3.3/1
1-yr dog (Beagle) OECD 452: Oral gavage at 0, 1, 3, and 6 mg/kg bw/day	Transient reduction in food consumption and weight	No adverse effect demonstrated	-	Stewart, 2010 (081055). B.6.3.4

4.7.1 Non-human information

Sub-chronic toxicity of Sulfoxaflor *via* the oral route was investigated in the mouse, rat and dog. A 28-day dermal toxicity study was conducted in the rat. The studies (excluding range-finding studies) are summarised in Table 17.

4.7.1.1 Repeated dose toxicity: oral

Repeated dose toxicity in the rat:

Study 1: 28 day dietary study

Test system/species/dose levels	**Significant Findings**	**NOAEL**	**LOAEL**	**Reference/ DAR reference**
28-d rat (F344/DuCrl) OECD 407: 0, 300, 1000, 2000 ppm, or	↓↓food consumption with body weight loss (3000 ppm)	300 ppm *equiv to* 24 mg/kg bw/day (males) and 26	1000 ppm *equiv. to* 79 mg/kg bw/day	Yano, *et al.*, 2009b (061170).

3000 ppm *equiv. to* 0, 24.8, 79.4, 155, or 205 mg/kg/day for males and 0, 26.5, 88.3, 170 or 192 mg/kg/day for females	-altered haematology: ↑haematocrit, reticulocyte counts, platelets. -↑ serum cholesterol and protein, ↓ ALP ↑Liver abs. and rel. wt -Hepatocellular hypertrophy, vacuolisation (fatty change) -Splenic erythroid extramedullary hematopoiesis	mg/kg bw/day (females)	(males) and 88 mg/kg bw/day (females)	B6.3.1/2

In a 28-day dietary toxicity study, groups of five male and five female Fischer 344 rats were given test diets formulated to supply 0, 300, 1000, 2000, or 3000 ppm Sulfoxaflor for 28 days. These dose levels corresponded to 0, 24.8, 79.4, 155, or 205 mg/kg/day for males and 0, 26.5, 88.3, 170 or 192 mg/kg/day for females, respectively. Toxicokinetic analysis of blood plasma was completed.

Administration of Sulfoxaflor to male and female rats at 3000 ppm resulted in excessive reductions in feed consumption (31% and 36%) and body weight loss (21% and 19%) compared to controls after 9 days of administration, respectively. The 3000 ppm dose groups were euthanized (day 9). The lower feed consumption was attributed to decreased palatability of rodent feed containing Sulfoxaflor and was responsible for the decreased body weights.

Animals in all other dose groups exhibited a dose-related decrease in feed consumption at the start of exposure, which was due to decreased palatability of treated diets containing Sulfoxaflor. Males given 300, 1000 or 2000 ppm consumed 5%, 29% or 54% less feed than control animals after one day. Females given 300, 1000, or 2000 ppm consumed 7%, 26% or 48% less than control animals. Feed consumption increased in animals given 1000 or 2000 ppm for the remainder of the study. Feed consumption for males given 300 or 1000 ppm was comparable to controls by the end of the study and was 6% decreased at 2000 ppm. Females given 300, 1000 or 2000 ppm consumed 8%, 6% or 11% less than controls at the end of the study.

Animals given 1000 or 2000 ppm had decreased body weight after one day of exposure, and was considered a secondary effect of the reduced feed consumption. By day 28, males and females given 2000 ppm weighed 8.5% and 10% less, respectively, than controls. Males and females given 300 or 1000 ppm had body weights comparable to controls on day 28.

Males and females (1000 or 2000 ppm) had dose related increases in serum total cholesterol levels, and both sexes given 1000 or 2000 ppm, also had total serum protein levels higher than controls. Albumin and globulin levels were higher than concurrent and historical controls and were considered to be treatment related in males given 1000 or 2000 ppm and females given 2000 ppm.

Males and females given 2000 ppm had treatment-related decreases in final body weights. Males and females given 1000 or 2000 ppm had increased absolute and relative liver weights that were dose and treatment related.

There were a number of organ weights of males and females given 2000 ppm that were altered and included: relative brain (males and females), relative kidney (males), relative testes (males), relative thyroid (males and females), absolute heart (males), and absolute spleen (males and females). These differences in organ weights were considered to be

secondary to the lower body weights of this dose group. This conclusion is supported by the absence of histopathological changes in these organs.

Treatment-related histological effects were observed in the livers of males and females given 1000 or 2000 ppm and consisted of a dose related increase in the severity (very slight to moderate) of hepatocellular hypertrophy, with altered tinctorial properties, involving the centrilobular to midzonal regions of the hepatic lobule. Effects were more prominent in males, compared to females, increasing to moderate severity in 2000 ppm males. Vacuolization, consistent with fatty change, involving hepatocytes primarily in the right lateral lobe was also occasionally seen in a multifocal distribution in males given 1000 or 2000 ppm and in one female given 2000 ppm. The restriction of this alteration to only one liver lobe, the minor nature of the effect (very slight or slight) and the lack of a clear dose response relationship in regards to severity suggests that this may not be a significant effect.

Toxicokinetic analysis of the plasma showed that levels of Sulfoxaflor (AUC24h) were effectively proportional to dose; 3.3 to 3.6 fold increase between 300 and 1000 ppm groups and a 2.0-fold increase between 1000 and 2000 ppm dose groups. Females were more efficient in eliminating the test material than males. The 24 hour systemic dose as measured by the AUC24h was 21, 15 and 14% lower in females than males at 300, 1000 and 2000 ppm dose groups, respectively (corresponding to 210, 693, 1371 µg h/ml versus 167, 591 and 1183 µg h/ml at the low, middle and high doses, respectively). Plasma elimination half-life of Sulfoxaflor in male rats was between 7 and 8 hours; whereas it was 32-43% lower in females (between 4-5 hours).

The no-observed-adverse-effect-level (NOAEL) for both sexes is 300 ppm (26.5 mg/kg/day) and the lowest-observed- adverse-effect-level (LOAEL) is 1000 ppm (88.3 mg/kg/day), based on enlarged livers (size and weight) with hepatocellular hypertrophy, slight vacuolization (males), and increased cholesterol levels.

Study 2: 90-day dietary study

Test system/species/dose levels	**Significant Findings**	**NOAEL**	**LOAEL**	**Reference/ DAR reference**
90-d rat (F344/DuCrl) OECD 424: 0, 100, 750, 1500 ppm, or 3000 ppm *equiv. to* 0, 6.36, 47.6, or 94.9 mg/kg/day for males and 0, 6.96, 51.6, or 101 mg/kg/day for females	-↓food consumption and weight gain -↑Liver wt -↑cholesterol. -liver histopath (hypertrophy, necrosis and vacuolisation)	100 ppm *equiv. to* 6.36 mg/kg bw/day	750 ppm *equiv. to* 47.6/51mg/kg bw/day	Yano, *et al*., 2009b (071057). B.6.3.2/1

In a 90-day oral toxicity study (DAR B.6.3.2/1) ten male and ten female Fischer 344 rats per group were given test diets formulated to supply 0, 100, 750 or 1500 ppm Sulfoxaflor (purity 96.6%; Lot # E2198-17, TSN106108) corresponding to time-weighted average concentrations of 0, 6.36, 47.6, or 94.9 mg/kg/day for males and 0, 6.96, 51.6, or 101 mg/kg/day for females, respectively. Parameters evaluated were daily cage-side observations, weekly detailed clinical observations, ophthalmic examinations, functional observational battery (FOB, pre-exposure and prior to necropsy, comprising cage-side, hand-held, and open field observations, rectal temperature, fore- and hindlimb grip performance, landing foot splay, and motor activity), body weights, feed consumption, prothrombin time, hematology, urinalysis, clinical chemistry, selected organ weights and gross and histopathologic examinations, which

included a specifically detailed review of the nervous system. The study also included integrated toxicokinetics and an assessment of immunotoxicity.

Males (all treated doses) and females (750 ppm and 1500 ppm) exhibited dose-related lower feed consumption, which was due to decreased palatability of diets containing Sulfoxaflor. During the first four days males given 100, 750 or 1500 ppm consumed 5%, 12% or 24% less feed than controls, and females given 750 or 1500 ppm consumed 8% or 21% less feed than controls. However, feed consumption for males at all dose levels was comparable to controls by the end of the study. Female feed consumption in the 750 and 1500 ppm groups was 5% or 8% lower than controls at 90 days, respectively, and statistically identified.

Male and females given 750 or 1500 ppm gained less weight than controls during the first four days of treatment and were dose related. After four days of treatment, body weight gains of males given 750 or 1500 ppm were 24% or 45% lower than controls, respectively, and body weight gains of females given 750 or 1500 ppm were 13% or 60% lower than controls, respectively. These animals gained weight for the remainder of the study. By day 90, males and females given 750 or 1500 ppm weighed 8% or 9% and 3% or 8% less than controls, respectively. By day 90, the body weight gain of males and females given 750 or 1500 ppm was 11% or 13% and 9% or 20% less than controls, respectively. All body weight effects were considered secondary to the lower feed consumption due to decreased palatability of the test material in the feed. Males and females given 100 ppm had body weights comparable to the controls at the end of the 90-day study.

Serum cholesterol levels in males and females given 750 or 1500 ppm had dose-related increases of 51% or 127% and 32% or 83% compared to controls, respectively. All other clinical pathology values were comparable to control values.

Rats given 750 or 1500 ppm had statistically identified higher absolute and relative liver weights that were dose related, and interpreted to be treatment related. The absolute liver weights of males and females given 750 or 1500 ppm was 6% or 5% and 25% or 17% higher than controls, respectively and the relative liver weights of males and females given 750 or 1500 ppm was 14% or 8% and 41% or 27% higher than controls, respectively. There were a number of additional differences in organ weights of males and females given 750 or 1500 ppm that were statistically identified. These differences in organ weights were secondary to the lower body weights of these dose groups and did not reflect a primary target organ effect of Sulfoxaflor. This conclusion was supported by the absence of histopathological changes in these organs.

Treatment-related histological liver effects occurred in males and females given 750 or 1500 ppm and consisted of a dose-related increase in the severity (slight to moderate) of hepatocellular hypertrophy (with altered tinctorial properties) involving the centrilobular to midzonal regions of the hepatic lobule. Individual hepatocyte necrosis was also observed in the centrilobular region with a multifocal distribution to a very slight or slight degree. All effects were seen in both sexes but were more prominent in males compared to females. Vacuolization of hepatocytes, consistent with fatty change, was also observed in all males in the 750 and 1500 ppm groups at very slight, slight or moderate degrees. In addition, in the rats with the greatest degree of hepatocellular hypertrophy, necrosis and vacuolization, there was an increase in the incidence of rats with multifocal aggregates of macrophages-histocytes. The microscopic changes were present in all three lobes of the liver examined in male and female rats; however, they were more readily apparent in the right lateral lobe. The microscopic changes in the liver were consistent with the increased liver weights and cholesterol levels noted for these rats.

There were no indications of neurotoxicity at any dose level. Assessment of immunotoxicity as measured by immune responsiveness in the sheep red blood cell antibody-forming cell assay indicated there was no effect on immune responsiveness in female rats up to and including the high dose level of 1500 ppm. There was no effect on immune responsiveness for male rats in the 100 and 750 ppm groups, while the 1500 ppm group displayed a lower, non-statistically significant, response (26% lower) when compared to the vehicle control group. The lower AFC response in the high dose male group coincided with considerable general toxicity, including decreased body weights and increases in liver weight (absolute and relative), hepatocellular hypertrophy, necrosis, vacuolization consistent with fatty change, multi-focal aggregates of macrophages, and elevated serum cholesterol, for which the overall NOEL was 100 ppm. Therefore, the lower AFC response in the high dose males was considered secondary to systemic toxicity and thus does not reflect primary immunotoxic potential for Sulfoxaflor.

The potential to recover from the effects induced by Sulfoxaflor was demonstrated in male and female rats given 0 or 1500 ppm for 90 days, followed by control feed for 28 days. Nearly complete recovery was seen in body weights (only ~5% lower for both sexes). Feed consumption during the 28-day recovery period was comparable to controls in both sexes. Also, the serum cholesterol levels that were elevated during the 90-day study in both males and females were normal following the 28-day recovery period and a complete recovery was seen in the absolute and relative liver weights of males and females given 1500 ppm.

There was partial recovery of the microscopic hepatic effects. Two male rats in the 1500 ppm group still had recognizable hepatocellular hypertrophy of a very slight degree in the centrilobular and midzonal regions. One of these two rats also had multifocal, very slight individual hepatocellular necrosis. Multifocal, very slight or slight, hepatocellular vacuolization consistent with fatty change was present in most of the recovery males; however, the degree of involvement was substantially less severe in the recovery group. There were no microscopic treatment-related changes present in the liver in females given 1500 ppm.

Toxicokinetic analysis of the plasma showed that the systemic exposure of Sulfoxaflor was dose proportional. An ~8-fold increase in AUC24h was found between 100 and 750 ppm groups and a ~2-fold increase between 750 and 1500 ppm doses. Females were more efficient at eliminating the test material from their system than males. The 24-hour systemic dose as measured by the plasma AUC24h was 15, 16 and 14 percent lower in females than males at 100, 750 and 1500 ppm nominal dose groups, respectively. Plasma elimination half-life of Sulfoxaflor in male rats was ~9 hours; whereas, it was ~8 hours in females (12% lower). The chromatograms of the plasma samples taken from Sulfoxaflor dosed rats (via diet) contained up to 5 minor peaks in addition to the parent compound. These peaks may represent metabolites of the test material or metabolites of test material impurities. Absolute quantitation of the minor metabolites could not be made, due to lack of reference standards. Elimination of Sulfoxaflor in urine over 24 hours ranged between 51 and 61% of the ingested dose, with the exception of high dose in males which was 37% of the ingested dose, 26 days after the initiation of the study. Elimination of test material in 0-24 hr urine on days 84 and 85 ranged between 52-69% for the lower two dose levels, but was somewhat lower at the high dose for both sexes (32-36% of ingested dose). In addition to parent Sulfoxaflor, four urinary metabolites were detected. One peak was a known impurity in this lot of the test material. No definitive quantitation of the other three metabolites was obtained.

In conclusion, during the first few days of the study decreased feed consumption and body weight gain was observed at all dose levels. Subsequently feed consumption and body weight

gain returned to normal in the 100 ppm group. There were no other observations noted in the parameters evaluated at this dose level. At 750 ppm and 1500 ppm the primary toxicologically significant findings were increased serum cholesterol levels, increased liver weight and histopathological findings in the liver. All of the effects due to ingestion of the test material had recovered during the 28-day recovery phase, except minor histopathologic liver effects noted only in males given 1500 ppm.

The lowest-observed-adverse-effect level (LOAEL) for systemic toxicity is based on the observation of decreased body weight, elevated cholesterol levels, hepatoxicity (increased weight, hypertrophy, necrosis, and vacuolization) at 750 ppm (47.6 mg/kg/day). The no-observed-adverse-effect-level (NOAEL) is 100 ppm (6.36 mg/kg/day).

There were no indications of neurotoxicity at any dose level, therefore, a LOAEL was not determined. The NOAEL is ≥ 1500 ppm (94.9 mg/kg/day) for neurotoxic effects.

There were no indication of immunotoxicity at any dose level, therefore, the LOAEL was not determined. The NOAEL is ≥ 1500 ppm (94.9 mg/kg/day) for immunotoxic effects.

Study 3: Rat 1-year dietary study (DAR B.6.5.1.1)

See Chronic Toxicity (4.10)

Repeated dose toxicity in the mouse:

Study 1: 28 day dietary study

Test system/species/dose levels	**Significant Findings**	**NOAEL**	**LOAEL**	**Reference/ DAR reference**
28-d mouse (Crl:CD1/(ICR) OECD 407: 0, 300, 1500 and 3500 ppm *equiv to* 0, 43.9/53, 230/273, 524/638 mg/kg bw/day (male/female)	-↑Liver wt -↑ALT, AST, triglyceride, ALP -Hepatocellular hypertrophy, fatty change, some necrosis (males), vacuolisation, mitotic figures -↑adrenal weight in males (abs and rel) -hypertrophy of the zona fasiculata of adrenal cortex (males)	300 ppm *equiv. to* 53 /43.9 mg/kg bw/day (males/females)	1500 ppm *equiv to* 230/273 mg/kg bw/day (males/females)	Thomas, *et al.*, 2008 (071053). B.6.3.1/3b

In a 28 day toxicity study in mice, groups of five male and five female CD-1 mice were fed diets supplying 0, 300, 1500, or 3500 ppm Sulfoxaflor, equivalent to 0, 44, 230, 524 mg/kg/day in males and 0, 53, 273, and 638 mg/kg/day in females, for at least 28 days. Parameters evaluated were daily cage-side observations, weekly detailed clinical observations, ophthalmic examinations, body weights, feed consumption, hematology, clinical chemistry, selected organ weights, and gross and histopathologic examinations.

Feed consumption values for males and females given 1500 or 3500 ppm were lower than the controls during days 1-2, whereas feed consumption values were comparable to the controls after day 4. Slight treatment-related decreases in body weights and body weight gains were observed in males and females given 1500 or 3500 ppm during the first week of the study, however, they were comparable to controls through the rest of the study.

Toxicokinetic analysis of the plasma showed that dose-proportional intake of Sulfoxaflor was translated into a dose-proportional increase in plasma concentrations of Sulfoxaflor. The systemic exposure of Sulfoxaflor was ~40% higher in males than in females. The 24-hour urine urinary elimination of Sulfoxaflor in males was between 33 and 44% and in females was between 23 and 65% of what they consumed during a 24-hour period, indicating that the majority of the dietary administered Sulfoxaflor was excreted essentially unchanged in the urine.

At termination on day 30, males and females given 1500 or 3500 ppm had treatment-related elevations in mean serum ALP, ALT, AST activities and triglycerides. There were treatment-related increases in the mean liver weights and liver histopathology of males and females given 1500 or 3500 ppm compared to controls. Males given 300 ppm had marginal treatment-related increases in liver weights over the respective control values, but were not associated with detectable hepatocyte hypertrophy or clinical chemistry changes and therefore, considered a non-adverse effect. Males given 3500 ppm had treatment-related elevations in absolute and relative adrenal gland weights that corresponded to hypertrophy of the zona fasciculata of the adrenal cortex. Males given 3500 ppm had treatment-related lower absolute and relative kidney weights.

In summary, due to higher systemic exposure in male mice, higher toxicity of Sulfoxaflor was observed.

The no-observed-adverse-effect-level (NOAEL) in male and female mice is 300 ppm (43.9 and 53 and mg/kg/day, respectively). The lowest-observed-adverse-effect-level (LOAEL) in male and female mice is 1500 ppm (30 and 273 mg/kg/day, respectively), based on increased liver weights, increased liver enzymes (ALT, AST), and hepatocellular hypertrophy in both sexes. Liver histopathology (necrosis) in male mice was also seen at this dose. At 3500 ppm (524 and 638 mg/kg/day, respectively), the same effects were seen along with increased triglycerides and ALP in both sexes, liver histopathology in males (mitotic cells, vacuolization/fatty change) and females (necrosis), and adrenal effects (increased weights and hypertrophy) in males.

This study is fully reliable (acceptable/guideline).

Study 2: 90-day dietary study

Test system/species/dose levels	**Significant Findings**	**NOAEL**	**LOAEL**	**Reference/ DAR reference**
90-d mouse (Crl:CD1/(ICR) OECD 408: males; 0, 100, 750 and 1250 ppm *equiv to* 0, 12.8, 98.0 or 166 mg/kg/day and females; 0, 100, 1500 or 3000 ppm *equiv to* 0, 16.2, 247 or 489 mg/kg/day	-↑Liver wt -↑ALT, AST, ALP, cholesterol, bilirubin -liver histopath (necrosis and fatty change) -↑adrenal weight -adrenal hypertrophy -haematopoiesis (spleen)	100 ppm (13 and 16 mg/kg bw/day)	Males: 750 ppm equiv to 98 mg/kg bw/day. Females: 1500 ppm equiv to 247 mg/kg bw/day	Thomas, *et al.*, 2010 (071162). B.6.3.2/2

In a 90 day oral toxicity study, groups of ten male and ten female CD-1 mice were fed diets supplying 0, 100, 750 or 1250 ppm and 0, 100, 1500, 3000 ppm Sulfoxaflor, respectively for at least 90 days. These concentrations supplied average doses of 0, 12.8, 98.0 or 166 mg/kg/day for males and 0, 16.2, 247 or 489 mg/kg/day for females. Parameters evaluated were: daily cage-side observations, weekly detailed clinical observations, ophthalmic examinations, body weights, feed consumption, hematology, clinical chemistry, selected

organ weights, and gross and histopathologic examinations. In addition, toxicokinetic analyses were conducted on urine (day 80) and terminal blood plasma.

There were no treatment-related effects on clinical signs, ophthalmic parameters, body weights or feed consumption.

Toxicokinetic analysis of the plasma showed that dose-proportional intake of Sulfoxaflor translated into a dose-proportional increase in plasma concentrations of Sulfoxaflor only up to the mid dose for both male (750 ppm, 92 mg/kg/day) and female (1500 ppm, 227 mg/kg/day) mice. In males, the systemic exposure, as measured by the plasma concentration of Sulfoxaflor, became supra-linear between the mid (92 mg/kg/day) and high (152 mg/kg/day) doses (3.9-fold increase instead of 1.6-fold expected from the difference in the test material intake between the mid and high doses). Plasma concentrations of Sulfoxaflor in females reached a plateau, remaining almost unchanged between the mid (227 mg/kg/day) and the high (467 mg/kg/day) doses. Total elimination of Sulfoxaflor in 24-hour urine remained dose-proportional only up to the mid dose and showed less than dose-proportional increase at the highest dose, both in male and female mice. These data are consistent with a saturation of elimination of Sulfoxaflor in male mice at the highest dose and a saturation of absorption of Sulfoxaflor from the gastrointestinal tract in female mice at the highest dose. On the basis of these results, the kinetically-derived maximum dose (KMD) *i.e*., the dose above which kinetics become non-linear was considered to be 92 (750 ppm) and 227 (1500 ppm) mg/kg/day for male and female mice, respectively.

Males given 750 or 1250 ppm and females given 1500 or 3000 ppm had treatment-related increases in absolute (20% and 74%; 36% and 54%, respectively) and relative liver weights (26% and 85%; 40% and 50%, respectively) compared to controls. Treatment-related organ weight changes consisted of increased absolute and relative adrenal weights in males given 1250 ppm, and lower absolute kidney weights in males given 750 or 1250 ppm. The lower kidney weights were however, considered non-adverse. Males given 1250 ppm had a treatment-related 200% increase in serum alanine aminotransferase (ALT), 43% increase in aspartate aminotransferase (AST) and 142% increase in alkaline phosphatase (ALP) activities. Serum total cholesterol and total bilirubin concentrations were decreased in males given 750 and 1250 ppm and were attributed to treatment. Females given 1500 or 3000 ppm had treatment-related elevations in serum ALT (125% and 171% increase, respectively) and AST activities (44% and 31% increase, respectively), and decreased ALP activity (3000 ppm only) compared to controls. Serum triglycerides were elevated in females given 1500 or 3000 ppm and serum cholesterol concentration was elevated in females given 3000 ppm, and attributed to treatment. There was a minor, treatment-related reduction in hematocrit and hemoglobin concentration in females given 1500 or 3000 ppm.

Males given 750 or 1250 ppm had slight or moderate, and females given 1500 or 3000 ppm had very slight, slight or moderate, treatment-related, centrilobular to midzonal hepatocyte hypertrophy with altered tinctorial properties. Other treatment-related histologic liver effects consisted of an overall, very slight or slight increase in the numbers of mitotic figures (hepatocytes in mitosis) in the liver of males given 1250 ppm, and very slight or slight fatty change in hepatocytes of males given 750 or 1250 ppm. Males given 750 or 1250 ppm had treatment-related, very slight or slight necrosis of scattered, individual hepatocytes, whereas this change in females given 1500 or 3000 ppm was infrequent or minimal. Males given 750 or 1250 ppm and females given 1500 or 3000 ppm had treatment-related, very slight hypertrophy of the zona fasciculata of the adrenal cortex. A very slight, treatment-related fatty change was also present in the zona fasciculata of the adrenal cortex in some females given 1500 or 3000 ppm. Four out of ten females given 3000 ppm had very slight, treatment-

related increase in extramedullary erythrocytic hematopoiesis in the spleen.

The No-Observed-Adverse-Effect Level (NOAEL) for males and females is 100 ppm (13 and 16 mg/kg/day, respectively). The Lowest-Observed-Adverse-Effect-Level (LOAEL) for males is 750 ppm (98 mg/kg/day), based on increased liver weights and hypertrophy, liver histopathology (necrosis/fatty change), increased cholesterol and bilirubin, and adrenal hypertrophy. At 1250 mg/kg/day (169 mg/kg/day) , the same effects were observed, along with increased liver enzymes (ALT, AST, ALP), mitotic figures (hepatocytes), and increased adrenal weights.

The LOAEL in females is 1500 ppm (247 mg/kg/day), based on increased liver weights, liver hypertrophy, increased liver enzymes (ALT, AST), increased triglycerides, increased hemoglobin and hematocrit, and adrenal changes (hypertrophy and fatty change/vacuolization). At 3000 mg/kg/day (489 mg/kg/day), the same effects were observed, along with decreased ALP, increased cholesterol, and hematopoiesis in the spleen. The toxicokinetics data indicates that there is a saturation of absorption from the intestinal tract at 1500 ppm in females and a maximal excretory level at 750 ppm in males. The study is fully reliable and satisfies the guideline requirement for a subchronic oral study (OPPTS 870.3100; OECD 408) in mice.

Repeated dose toxicity in the dog:

Study 1: 28 day probe and gavage study

Test system/species/dose levels	Significant Findings	NOAEL	LOAEL	Reference/ DAR reference
Palatability probe/beagle dog/(dietary/capsule/gavage)/500 ppm or 15 mg/kg.bw/day	Oral gavage exposure at 15 mg/kg/day technical grade Sulfoxaflor was tolerable route and concentration for the test material, as determined by adequate food consumption and tolerable in-life clinical signs.	Not applicable	Not applicable	Stewart, (2009). DAR B.6.3.1/4

In a 28-day oral palatability probe study Sulfoxaflor (XDE-208 technical grade) was administered to Beagle dogs via dietary, capsule or oral gavage administration for up to 28 days. Using the same animals (with 1 or 2-week breaks of basal diet only between dosing regimens), routes of test material administration and dosing regimens were altered for a total of five dosing groups, during the course of the study as follows. Three female dogs received technical grade test material via the diet, ad libitum, for 6 consecutive days at a dose level of 500 ppm (Group 1). The dosing route for these animals was changed to analytical grade test material via oral gavage, once daily for 28 consecutive days, at a dose level of 15 mg/kg/day (Group 3). Another group of three female dogs received the technical test material via capsule, twice daily for 6 consecutive days, at a dose level of 15 mg/kg/day (Group 2). The dosing route for these animals was changed to dietary, ad libitum (technical grade) for 5 consecutive days, at a dose level of 100 ppm (Group 4). The dosing route for these animals was once again changed to oral gavage (technical grade), once daily for 28 consecutive days, at a dose level of 15 mg/kg/day (Group 5). For both oral gavage groups, the vehicle was 0.5% methylcellulose in deionized water and the dose volume was 10 mL/kg. A previous, preliminary palatability probe study (MRID 47832056) did not identify adequate oral

acceptance by diet and capsule administration.

Observations for morbidity, mortality, injury and the availability of food and water were conducted twice daily for all animals. Clinical observations were conducted daily. Body weights and food consumption were measured and recorded daily. Blood samples for clinical pathology evaluations were collected from all animals pretest and on Days 6 and 29 (prior to necropsy), and urine samples were collected at necropsy from all animals on Day 29. Blood samples for determination of the plasma concentrations of the test article were collected from animals in Group 4 at designated time points on Day 5 (the last day of dietary dosing), and from animals in Groups 3 and 5 at designated time points on each respective Day 28 (prior to necropsy). At study termination, necropsy examinations were performed and organ weights were recorded. A complete set of tissues from Group 5 animals was sent to the Sponsor for microscopic evaluation. Findings were compared with historical range data due to the lack of concurrent controls.

All animals survived until the scheduled termination intervals. However, there were clinical findings and body weight findings that were associated with reduced food consumption (50% or more compared to pretreatment). In some instances, the reduced food consumption was accompanied by a lack of sufficient fluid intake which resulted in a loss of skin elasticity. In addition, intermittent emesis and faecal alterations (discolored, mucoidal, and soft/watery faeces) was likely test material-related.

Oral gavage administration at 15 mg/kg/day was a tolerable route and concentration for the technical grade test material exposure as determined by adequate food consumption and tolerable in-life clinical signs. Exposure at 100 ppm *via* dietary route was well tolerated as determined by adequate food consumption, but the amount of test material consumed was not sufficient to justify it as a potential highest dose for subsequent studies. Exposure at 500 ppm *via* dietary route or 15 mg/kg/day via capsule route was not well tolerated as determined by insufficient food consumption. Therefore, the dietary route could not be considered a viable method of test material exposure over a sustained duration. Based on this information, oral gavage exposure was determined to be the most appropriate route for a sustained duration of test material exposure in Beagle dogs.

This non-guideline study is acceptable. The study is not GLP compliant. However, all experiments were done according to GLP standards. It was conducted to determine palatability and the appropriate method of oral administration for sub-chronic and chronic oral toxicity studies and not to satisfy guideline requirements.

Study 2: 90-day gavage study

Test system/species/dose levels	Significant Findings	NOAEL	LOAEL	Reference/ DAR reference
90-d dog (Beagle) OECD 409: Oral gavage at 0, 1, 3, and 10 (days 1-5)/6 mg/kg bw/day (days 5-90)	↓↓food consumption, weight loss and reduced weight gain ↑Liver wt	6	10	Stewart, 2010 (081054). B.6.3.3/1

In a 90 day oral dog study Sulfoxaflor was administered by gavage to 4 beagle dogs/sex/dose at dose levels of 0 (vehicle only), 1, 3 or 10 mg/kg/day. The 10 mg/kg/day dose level was reduced to 6 mg/kg/day on Day 5 of the study due to intolerance (lack of food consumption). Controls received the vehicle, 0.5% methylcellulose (Methocel A4C) in water. The test

material or vehicle was given once a day for 90 consecutive days, at a dose volume of 10 mL/kg.

Observations for morbidity, mortality, injury, availability of food and water were conducted twice daily. Clinical observations were recorded weekly. Food consumption was measured daily from Days 1 to 7 and 9 to 35, twice weekly from Week 6 to 12, and once during Week 13. Blood and urine samples were collected from all animals pretest and during Weeks 6 and 13. Blood and urine samples were collected from all animals at intervals during Week 13 for plasma and urine concentrations of the test material. At termination, necropsies were performed, organ weights recorded, and selected tissues were microscopically examined.

All animals survived the study and there were no treatment-related clinical signs. Treatment-related decreased mean body weights were observed at the high dose level of 10 mg/kg/day in both sexes, primarily during the first week of exposure. However, mean body weights at this exposure level did not have any significant decreases beyond Week 2, after the dose was reduced to 6 mg/kg/day on Day 5. By approximately Week 9, the mean body weights at this exposure level had returned to pre-exposure values. The transient nature of the decreased mean body weights was a clear indication that the effect was related to exposure at 10 mg/kg/day and not related to exposure at 6 mg/kg/day.

Sulfoxaflor at 10 mg/kg/day was not tolerated, as determined by significant and unacceptable decreases in food consumption. However, reduction to 6 mg/kg/day after 5 days allowed for food consumption in the affected group comparable to controls for the remainder of the study. Treatment-related, decreased food consumption values at high dose were observed in males and females during the first 2 weeks of the study. By Week 3, the mean food consumption values had stabilized and were similar to control values with males appearing to recover slightly sooner than females. Based on the data, the effect on food consumption was considered to be related to exposure at 10 mg/kg/day, with residual effects prolonging the instability in several animals into Week 3 of the study. There were no other notable food consumption findings related to intake of the test material for the remainder of the study. There were no other parameters under evaluation that showed treatment-related effects.

In Week 13, the steady-state systemic dose (AUC24 h) of Sulfoxaflor to dogs after doses of 1, 3, 6 mg/kg/day was 32 ± 6, 84 ± 23 and 147 ± 13 µg*h/ml in males and 22 ± 3, 71 ± 26 and 119 ± 19 µg*h/ml in females, respectively. The increase in systemic dose was dose-proportional in female dogs and was approximately dose proportional in male dogs across all three dose levels. The dose-corrected AUC24 after oral gavage to dogs was 3 to 4 fold higher than that observed in dogs after 28 days of oral gavage and 3 to 5-fold higher than that observed in rats after 90 days of dietary exposure. Urinary elimination of Sulfoxaflor was 70 ± 6, 76 ± 12, and 59 ± 33 percent of administered dose in male and 69 ± 4, 80 ± 6, and 74 ± 13 percent in female dogs at 1, 3, and 6 mg/kg/day doses, respectively. The mean plasma elimination half-life of XDE-208 in Week 13 of dosing was between 17 and 28 hours.

The LOAEL is 10 mg/kg/day, based on excessively reduced food consumption and body weight loss prior to reduction of the dose on Day 5. The NOAEL is 6 mg/kg/day.

This study is acceptable and satisfies the guideline requirement for a sub-chronic oral toxicity (oral gavage) - Beagle dogs (OPPTS 870.3150 (non-rodent); OECD 409; EEC Part B.27; JMAFF Sub-chronic Oral Toxicity Study).

Study 3: 1-year gavage study

Test system/species/dose levels	Significant Findings	NOAEL	LOAEL	Reference/ DAR reference
1-yr dog (Beagle) OECD 452: Oral gavage at 0, 1, 3, and 6 mg/kg bw/day	Transient reduction in food consumption and weight	No adverse effect demonstrated	-	Stewart, 2010 (081055). B.6.3.4

In a chronic oral toxicity study, four Beagle dogs/sex/dose were administered Sulfoxaflor at doses of 0, 1, 3 or 6 mg/kg/day for 52 weeks. Test material was administered by a daily gavage dose in 0.5% aqueous methylcellulose vehicle (Methocel A4C; dosing volume 10 mL/kg). Dietary or capsule administration was not performed due to unsatisfactory food consumption using these methods. Observations for morbidity, mortality, injury and the availability of food and water were conducted twice daily for all animals. Toxicity was assessed by weekly detailed clinical observations, food consumption and body weight measurements, ophthalmoscopic examinations, and clinical pathology evaluations. Blood and urine samples for determination of plasma concentrations of the test material were collected from all animals at Weeks 13, 26 and 52. Toxicokinetic (TK) parameters were determined for the test article from concentration-time data. At study termination, necropsy examinations were performed, select organ weights were recorded, and tissues were preserved for subsequent microscopic examination.

No treatment-related deaths occurred. The increased frequency of soft and/or watery faeces in two males at 6 mg/kg/day was considered treatment-related due to the high incidence in these animals relative to other groups, but was not considered adverse because these findings had no effect on food consumption or body weight/weight gain. A transient, treatment-related decrease in food consumption and body weight was observed in females at 6 mg/kg/day during the first two weeks of dosing. The decreases in food consumption and body weight were considered non-adverse findings due to their transient nature. No definitive effects on hematological or clinical pathology parameters were noted. The systemic exposure of Sulfoxaflor (AUC_{24h}) in plasma was proportional across all dose levels in both sexes. With the exception of females at 52 weeks of exposure (urine values of high dose females fell below the regression line fitted to the mean values), toxicokinetic analysis of parent in urine showed that the systemic exposure of Sulfoxaflor (AUC_{24h}) was proportional across all dose levels and time points. There were no treatment-related organ weight changes or gross/histopathologic effects at any dose. The NOAEL is 6 mg/kg/day (soft/watery faeces in males and a transient decrease in food consumption and body weight in females during the initial two weeks were considered treatment-related, but not adverse). A LOAEL was not identified (> 6 mg/kg/day).

This study is classified fully reliable and satisfies the guideline requirement for a chronic oral toxicity study in the dog (OPPTS 870.4100; OECD 452).

4.7.1.2 Repeated dose toxicity: inhalation

No data available.

4.7.1.3 Repeated dose toxicity: dermal

28 day dermal study

Test system/species/dose levels	Significant Findings	NOAEL	LOAEL	Reference/ DAR reference
28-d rat dermal (F344/DuCrl) OECD 410: 0, 100, 500 and 1000 mg/kg bw/day semi-occluded	-↑Cholesterol (males) -slight ↑liver wt (slight) -liver hypertrophy (slight)	1000 mg/kg bw/day	Not determined	Thomas, 2009 B.6.3.7

In a repeated-dose dermal toxicity study Sulfoxaflor was applied dermally to; 10 Fischer DuCrl 344 rats/sex/dose, exposed at a semi-occluded, shaved skin test site to 0, 100, 500 or 1000 mg/kg body weight/day for six hours per day for 28 consecutive days. Parameters evaluated were daily cage-side and weekly detailed clinical observations, dermal observations, ophthalmic examinations, body weight, feed consumption, hematology, clinical chemistry, urinalysis, toxicokinetics of blood plasma, selected organ weights, and gross and histopathologic examinations.

Systemic toxicity: At 1000 mg/kg/day, males showed marginal increases in absolute and relative liver weights (6.5% and 4.4%, respectively above controls, $p<0.05$). A treatment-related histopathologic change was observed in the livers of 6 of 10 males, consisting of very slight hepatocyte hypertrophy, with altered tinctorial properties (increased cytoplasmic eosinophilia), involving the centrilobular/midzonal regions of the hepatic lobule. These were minor changes, not associated with increases in liver enzymes in the blood and hence, considered adaptive and non-adverse. Mean serum cholesterol was 17% higher compared to controls. This effect was considered treatment related but non-adverse because it was within the laboratory's historical control range. Toxicokinetic analysis showed that the average plasma concentration of test material at high dose (1000 mg/kg/day) was greater than dose proportional. Similar plasma concentrations were found prior to and 16 hr after test material removal, indicating some retention of test material at the application site. There were no treatment-related systemic effects observed in females at any dose. The NOAEL for systemic toxicity is ≥1000 mg/kg/day (mild liver effects observed in males indicated some dermal absorption, but were not considered adverse). A LOAEL for systemic toxicity was not determined (>1000 mg/kg/day).

Local dermal toxicity: There were no treatment-related gross or microscopic dermal effects at the application site. The NOAEL for local dermal toxicity is ≥1000 mg/kg/day. A LOAEL for local dermal toxicity was not determined (>1000 mg/kg/day).

4.7.1.4 Repeated dose toxicity: other routes

No data available.

4.7.2 Human information

No data available.

4.7.3 Other relevant information

No other relevant information.

4.7.4 Summary and discussion of repeated dose toxicity findings relevant for classification according to DSD

Significant palatability issues influenced the dose levels used in the investigation of sub-chronic in mice, rats and especially dogs. The dietary route was used for mice and rats while gavage was the only route feasible in the dog. Food consumption and body weight was affected at higher doses in all species. In short term (28-day & 90-day) dietary toxicity studies in rats and mice, the main target organ was the liver)(see DAR section B6.3.1- B6.3.2 for detailed analysis), with significant increases in liver weight at high dose levels in rats and mice from 750 ppm. Males were affected more than females, which may, at least in part, have been related to the initial longer half-life of elimination in males. The main effects observed in all of these studies at the LOAEL comprised a consistent pattern of increased liver weight with histopathologic effects including hepatocellular hypertrophy with altered tinctorial properties. In rats, single cell necrosis was detected at 90 days, with fatty change in males. In mice, hepatocellular necrosis was seen at 28 days in males, together with mitotic figures. Cholesterol levels were increased in rats but not in mice, which had elevated triglyceride in females. Also noted in mice was hypertrophy/vacuolisation of the *zona fasciculata* of the adrenal gland of both sexes and altered haematological parameters and some extramedullary haematopoiesis at high doses. Any alterations to other organs/tissues were within historical controls, lacked a dose response and were interpreted not to be of toxicological significance. There was no evidence of immunotoxicity or neurotoxicity seen in specific rat studies to examine these endpoints. A slight increase (statistically significant) in absolute and relative testis weight was considered related to the significantly adverse body weight effects at this dose level, was without histological correlate, and therefore not considered as toxicologically significant.

In the 1-year chronic toxicity in rats, adverse effects were limited to high dose (500 ppm/21.3 mg/kg bw/day) level males and females and comprised reduced body weight gain for females and increased blood cholesterol and liver effects comprising increased weight, hepatocellular hypertrophy, fatty change, single cell necrosis and increased aggregates of macrophages.

In the dog, gavage administration gave the highest achievable doses but the only effects were decreases in feed consumption and body weight gain at the highest dose tested.

4.7.5 Summary and discussion of repeated dose toxicity findings relevant for classification according to CLP

The lowest dose with adverse effect (liver) was 750 ppm *equiv. to* 47.6/51mg/kg bw/day from the 90-day rat study (Yano, *et al*., 2009). At this study, there was a dose-related and statistically significant increase in relative and absolute liver weight in both sexes. This was associated with statistically significantly increased hepatocellular hypertrophy and in addition, individual cell necrosis and vacuolisation occurred in males from 750 ppm. Clinical chemistry endpoints indicative of liver toxicity were altered at 1500 ppm and not 750 ppm. In general, the male rat was most sensitive to the liver effects.

A statistically significant increased spleen weight was noted in males at 750 ppm which may have been related to a decrease in body weight and was associated with some degree of splenic congestion. Splenic congestion was apparent in controls and some treated groups. Overall the spleen effects were of equivocal toxicological relevance. Following the 28-day recovery period (94.9 mg/kg bw/day only), the liver was normal in the females and adverse effects were significantly less in the liver of males. Some hepatocellular hypertrophy and

vacuolisation were still present males, though greatly reduced. It is reasonable to assume that the lesser effects seen at ≈ 50 mg/kg would also have recovered.

4.7.6 Comparison with CLP and DSD classification criteria

The evidence of this study indicates that the effects on the liver at approximately 50 mg/kg bw/day were slight to moderate adaptive change with some toxicity. The effects were shown to be significantly recovered following withdrawal of exposure to the higher dose level of 94.9 mg/kg bw/day. The cut-off value for classification with R48 according to the DSD is ≤ 50 mg/kg bw/day and associated with major functional change and/or major organ damage which the evidence suggests to be irreversible.

The above data do not meet the criteria for classification under DSD.

4.7.7 Conclusions on classification and labelling (STOT RE)

The evidence of this study indicates that the effects on the liver at approximately 50 mg/kg bw/day were slight to moderate adaptive change with some toxicity. The effects were shown to be significantly recovered following withdrawal of exposure to the higher dose level of 94.9 mg/kg bw/day.

The cut-off value for classification with STOT RE2 according to the CLP Regulation is 10 < C ≤ 100 mg/kg bw/day. STOT RE is assigned on the basis of 'significant' or 'severe' toxicity which causes functional disturbance or morphological change which are toxicological relevant. The increased liver size at ≈ 50 mg/kg bw/day is not associated with evidence of functional change at this dose. While there is evidence of liver toxicity (necrosis/fatty change without clinical chemistry), this is shown to recover in the higher dose level and is not considered severe.

Classification is not required under CLP.

4.8 SPECIFIC TARGET ORGAN TOXICITY (CLP REGULATION) – REPEATED EXPOSURE (STOT RE)

Relevant discussions on repeated dose toxicity based on short term and sub-chronic studies are found under sections 4.7.4 to 4.7.7.

4.8.1 Summary and discussion of repeated dose toxicity findings relevant for classification as STOT RE according to CLP Regulation

Long-term and carcinogenic life time studies using rats and mice show that the liver is the main target organ of sulfoxaflor (section 4.10.1). There is mechanistic evidence for a phenobarbital-type, CAR-mediated mechanism to explain the liver responses and enzyme induction profiles that occur with sulfoxaflor treatment (section 4.10.3). The observed liver tumours are species specific but the non-neoplastic liver effects (increase in liver weight and liver histopathology in the rat 2-year and mouse 18-month studies) are potentially relevant effects for humans.

Briefly, in the rat 2 year study (study 1, section 4.10.1.1), the liver was the primary target organ for histopathological changes in high dose males and females at 12 and 24 months. The absolute and relative liver weights for high-dose males (21.3 mg/kg bw/day, +17 % and +13%

respectively) and females (39.0 mg/kg bw/day, +3% and +6% respectively) were only significantly increased at the 12 month interim sacrifice and decreased thereafter for male high dose animals (liver absolute weight: -1%, and relative liver weight: +3% with respect to concurrent controls) and high dose females (liver absolute weight : +1 %, and relative liver weight: +8.3% with respect to concurrent controls), at the time of final sacrifice (table 4.10.1.1 Study 1.7). Non-neoplastic liver effects at 12 and 24 months consisted of slight hypertrophy of centrilobular and midzonal hepatocytes, increased incidence of very slight multifocal individual cell necrosis of centrilobular hepatocytes, and very slight multifocal aggregates of macrophages, and vacuolisation (consistent with fatty change) of hepatocytes in the high dose group.

In the mouse 18 month study (study 2, section 4.10.1.1), the liver was also the primary target organ and showed non-neoplastic, treatment-related liver effects at the highest dose (79.6 mg/kg bw/day), which included massively increased liver weights (greater than 79%), increased incidence of liver nodules, liver hypertrophy, and liver histopathology (necrosis, fatty change).

4.8.2 Comparison with criteria of repeated dose toxicity findings relevant for classification as STOT RE

The lowest dose with adverse effects (liver) was 500 ppm *equiv. to* 21.3mg/kg bw/day from the 2-year rat study (Stebbins, *et al*., 2010). In general, the male rat was the most sensitive sex to the liver effects.

The cut-off value for classification with STOT RE2 according to the CLP Regulation is $10 < C \leq 100$ mg/kg bw/day based on a 90-day rat study. STOT RE is assigned on the basis of 'significant' or 'severe' toxicity which causes functional disturbance or morphological change which are toxicologically relevant.

The guidance values specified according to CLP can be used as a basis to extrapolate equivalent guidance values for toxicity studies of greater (or lesser) duration than 90 days. The adjustments, using dose/exposure time extrapolation similar to Harber's rule would result in an approximate 8-fold reduction of the standard guidance values, (2 years = 730 days; $730/90 \approx 8$) giving an equivalent cut-off value for classification with STOT RE2 of $2 < C \leq 12.5$ mg/kg bw/day based on a 2-year rat study.

The variations in liver size and histopathological effects occur at ≈ 21 mg/kg bw/day which is in excess of the calculated guidance values for STOT-RE2. Classification is not required under CLP.

The cut-off value for classification with R48 according to the DSD is ≤ 50 mg/kg bw/day for a rat 90 day study and associated with major functional change and/or major organ damage which the evidence suggests to be irreversible. There is no indication of a major functional change in the rat studies and applying similar logic as with the CLP equivalent guidance values would result in ≤ 6.25 mg/kg bw/day for a rat 2-year study. The effects in the mouse 18-month (1.5 year) study are seen at ≈ 80 mg/kg bw/day and also would not trigger classification.

4.8.3 Conclusions on classification and labelling of repeated dose toxicity findings relevant for classification as STOT RE

Liver is the target organ in rodents but the effects are not severe enough at a low dose level to warrant classification.

4.9 Germ cell mutagenicity (Mutagenicity)

Table 18: Summary table of relevant *in vitro* and *in vivo* mutagenicity studies

Method	Test system	Results	Reference/ DAR Reference
Bacterial Reverse Mutation	*S. typhimurium E. coli*	Negative ±S9	Mecchi, 2007 B.6.4.1/1
In Vitro Mammalian Chromosome Aberration	Rat lymphocytes	Negative ±S9	Schisler *et al.*, 2007a B.6.4.1/2
In vitro Mammalian Cell Gene Mutation	Chinese hamster ovary cells CHO/HGPRT	Negative ±S9	Schisler *et al.*, 2007b B.6.4.1/3
Mammalian Erythrocyte Micronucleus	Mouse bone marrow polychromatic erythrocytes	Negative ±S9	LeBaron and Schisler, 2009 B.6.4.2

4.9.1 Non-human information

4.9.1.1 *In vitro* data

Study 1:

Method	Test system	Results	Reference/ DAR Reference
Bacterial Reverse Mutation	*S. typhimurium E. coli*	Negative ±S9	Mecchi, 2007 B.6.4.1/1

In independent trials of a reverse gene mutation assay (Sulfoxaflor Purity 96.6%; Lot/Batch No E2198-17) was prepared in dimethyl sulfoxide (DMSO) and tested in 4 strains of S. typhimurium (TA100, TA98, TA1535 and TA1537) and in E. coli WP2uvrA in a pre-incubation reverse mutation assay at concentrations ranging from 100 to 5000 µg/plate (both trials) with and without S9 activation. The S9 fraction was derived from the livers of male Sprague-Dawley rats induced with Aroclor 1254.

In both assays, the test material was not cytotoxic or mutagenic for any strain at concentrations up to the limit dose for this test system either in the absence or presence of S9 activation. The numbers of revertant colonies in all strain-specific positive control groups were clearly increased.

Under the conditions of this study, Sulfoxaflor did not induce gene mutation in any of the strains employed, either with or without metabolic activation, at concentrations up to the limit dose for this test system.

The study is classified as fully reliable (acceptable/guideline) and satisfies the guideline requirements (OPPTS 870.5100; OECD 471) for in vitro mutagenicity (bacterial reverse gene mutation) data.

Study 2:

Method	Test system	Results	Reference/ DAR Reference
In Vitro Mammalian Chromosome Aberration	Rat lymphocytes	Negative ±S9	Schisler *et al.*, 2007a (071029) B.6.4.1/2

In an *in vitro* chromosome aberration test, primary rat lymphocytes, derived from 10-11 week old Sprague-Dawley male rats, were exposed to Sulfoxaflor (Purity 96.6% Batch/Lot No. E2198-17, TSN106108) prepared in dimethyl sulfoxide (DMSO) at 0, 86.7, 173.3, 346.6, 693.3, 1389.5, and 2773 µg/mL without and with S9 activation for 4 hours and continuously for 24 hours at 0, 21.7, 43.3, 86.7, 173.3, 346.6, 693.3, 1386.5 and 2773 µg/mL without S9. The highest concentration tested approximates the limit dose of 10 mM and the S9 liver homogenate was prepared from Aroclor 1254-induced male Sprague-Dawley rats. Base on the analysis of mitotic indices (MIs), cultures treated for 4 hours with 0, 693.3, 1386.5, and 2773 µg/mL +/-S9 and cultures treated continuously for 24 hours with 173.3, 346.6 and 693.3 µg/mL –S9 were scored for structural and numerical chromosome aberrations.

Relative MIs (RMIs) were≥ 50% of control at 2773 µg/mL –S9 (4-hour treatment); 2200 µg/mL +S9 (4-hour treatment); and 346.6 µg/mL –S9 (24-hour treatment). There were no significant increases in the frequencies of cells with aberrations in either the presence or absence of S9 activation. Cultures treated with the positive control chemicals (mitomycin C without S9 and cyclophosphamide with S9) had significantly higher incidences of abnormal cells in all assays. Based upon these results, Sulfoxaflor was not considered to be clastogenic in this *in vitro* chromosomal aberration assay utilising rat lymphocytes.

This study is classified as totally reliable (acceptable/guideline) and satisfies the guideline requirement (OPPTS 870.5375, OECD 473) for *in vitro* mammalian cytogenetics (chromosome aberrations) data.

Study 3:

Method	Test system	Results	Reference/ DAR Reference
In vitro Mammalian Cell Gene Mutation	Chinese hamster ovary cells CHO/HGPRT	Negative ±S9	Schisler *et al.*, 2007b (071030) B.6.4.1/3

In independent trials of a mammalian cell forward gene mutation assay, Chinese hamster ovary (CHO/HGPRT) cells were exposed to Sulfoxaflor (Purity 96.6% Lot # E2198-17, TSN106108), prepared in dimethyl sulfoxide (DMSO), at concentrations ranging from 173.3 to 2773 µg/ml in the absence and presence of S9. The S9 liver homogenate was derived from the livers of Sprague-Dawley rats induced with Aroclor 1254. The highest concentration was the 10 mM limit for the assay system. Positive control chemicals used in this assay were ethyl methanesulfonate (EMS) in the absence of S9 and 20-methylcholanthrene (20-MCA) in the presence of S9.

Sulfoxaflor was tested up to the limit dose for this test system and failed to induce either a cytotoxic or mutagenic effect in either the absence or the presence of S9 activation. The

expected responses were obtained with the negative and positive controls either with or without S9 activation. It was, therefore, concluded that Sulfoxaflor was not active in this test system.

This study is classified as fully reliable (acceptable/guideline) and satisfies the guideline requirement (OPPTS 870.5300, OECD 476) for in vitro mutagenicity (mammalian forward gene mutation) data.

4.9.1.2 *In vivo* data

Study 1:

Method	**Test system**	**Results**	**Reference/ DAR Reference**
Mammalian Erythrocyte Micronucleus	Mouse bone marrow polychromatic erythrocytes	Negative ±S9	LeBaron and Schisler, 2009 (071100) B.6.4.2

In a bone marrow micronucleus assay, groups of 6 male and 6 female CD-1 mice were treated orally, by gavage, with 0, 100, 200 or 400 mg/kg/day of Sulfoxaflor (Purity 95.6%; Lot/Batch No. E2162-34, TSN003725-0001) prepared in METHOCEL™ on 2 consecutive days. The highest dose level of 400 mg/kg bw was selected for the main assay based on the results of a range-finding test in which doses≥1000 mg/kg bw/day caused more than 50% mortality in both sexes and 500 mg/kg bw/day caused unacceptable body temperature decreases in males only. Therefore, both sexes were evaluated in the main study. Groups of animals were sacrificed at 24 hours after the second treatment for the collection of femoral bone marrow and evaluation of polychromatic erythrocytes (PCE, 2000 PCE/animal) with micronuclei (MN-PCE) from the first five animals in each group. The proportion of PCE was determined based upon 200 erythrocytes per animal and the results expressed as a percentage. Mice treated with 120-mg/kg bw cyclophosphamide monohydrate by a single oral gavage dose and sacrificed at 24 hours served as the positive control.

All animals survived to the end of the observation period. Treatment related clinical signs (decreased activity) occurred in 3/6 male mice at 400 mg/kg /day and two of these mice also had body temperature decreases of up to 5.8°C five hours post-dosing. There were no statistically significant increases in the frequencies of MN-PCE in groups treated with the test material as compared to the negative controls. There were no statistically significant differences in the percent PCE in groups treated with the test material compared to negative controls. By contrast, a significant increase in the frequency of MN-PCE and a significant decrease in the relative proportion of PCE: NCE ($p<0.05$) was seen in the positive control group as compared to the negative control group. Under the experimental conditions used, Sulfoxaflor was not genotoxic in the mouse bone marrow micronucleus test.

This study is fully reliable (acceptable/guideline) and satisfies the guideline requirement (USEPA OPPTS 870.5395; OECD 474) for an in vivo mammalian cytogenetics – micronucleus assay in mice.

4.9.2 Human information

No data.

4.9.3 Other relevant information

None relevant

4.9.4 Summary and discussion of mutagenicity

A battery of *in vitro* genotoxicity studies – the Ames test, lymphocyte chromosome aberration test, and CHO-HGPRT test - showed that Sulfoxaflor does not cause gene mutations or chromosome aberrations. Additionally, an *in vivo* mouse micronucleus test showed that Sulfoxaflor does not induce micronuclei in somatic cells.

4.9.5 Comparison with CLP and DSD classification criteria

All tests were negative.

4.9.6 Conclusions on classification and labelling

Classification is not required.

4.10 Carcinogenicity

(Sulfoxaflor DAR, Chronic Toxicity section B.6.5)

There is one carcinogenicity study available in the rat (DAR section B.6.5.1.1) and one study available in the mouse (DAR section B.6.5.2.1). There was evidence of treatment related tumours found in the liver for rats and mice, increased Leydig cell tumour load in rats and an apparent increased incidence in rat preputial gland tumours. There were also numerous mode of action studies conducted and in addition a Human Relevance Framework (HRF) analysis for each tumour type to investigate the mechanisms behind the carcinogenic effects and show if these effects were relevant to human health and risk assessment.

Table 19: Summary table of relevant carcinogenicity studies

Method	Results	Remarks	Reference/ DAR reference
Rat (Fischer 344) 2-year combined toxicity and carcinogenicity dietary study. OECD, Guideline 453 (1981).	NOAEL: 100ppm (4.24mg/kg bw/day).	♂: 0, 25, 100, 500ppm equivalent to 0, 1.04, 4.24, and 21.3mg/kw bw/day respectively. 12 month interim sacrifice and end of study: Liver - increased blood cholesterol, liver weight, hypertrophy, fatty change, single cell necrosis and macrophages. End of study: Increased testes weight due to larger Leydig cell adenomas; secondary effects included atrophy of seminiferous tubules, reduced sperm in epididymides and secretory material in accessory sex glands. High dose: increased incidence and size of Leydig cell adenomas with secondary effects including preputial gland tumours; liver adenomas.	*Stebbins et al. 2010* (071187) (DAR B.6.5.1.1)
Mouse (CD1) 18-month carcinogenicity dietary study. OECD, Guideline 451 (1981).	NOAEL: 100ppm (10.4mg/kg bw/day).	♂: 0, 25, 100, 750ppm equivalent to 0, 2.54, 10.4, 79.6mg/kg bw/day Liver – adenomas and carcinomas; Increased liver weight, hypertrophy with eosinophilia, fatty change, single cell necrosis, eosinophilic/ vacuolated foci, mitosis.	*Thomas et al. 2010b* (081102) DAR B.6.5.2.1

Table 20a: Summary table of mechanistics / Mode of Action (MoA) studies

MoA Study: *Ex vivo* gene expression and cell proliferation analyses in rats and mice.	Mouse/CD1 (♀) and Rat/F344 (♂ and ♀) Dose: Mice: 0, 3000, 4500ppm. Rats: 0, 2000ppm	DAR section B.6.5.3.1 Sulfoxaflor-induced gene expression profile in mice and liver (hepatocellular) proliferation in both mice and rats characteristic of phenobarbital-like CAR agonism.	*Geter & Kan 2008* (081102)
MoA Study: Targeted gene expression, cell proliferation and cytochrome P450 enzymatic activity in rats.	Rat/F344 (♂ and ♀) Dose: 0, 100, 750, 1500ppm for 3 or 7 days.	DAR section B.6.5.3.2 Sulfoxaflor-induced liver effects were PB-like. Males were affected more than females. Neither AhR nor PPARα were involved.	*Geter & Card 2010* (070339)
MoA Study: Mode of Action Study Investigating Liver Weight Effects in Crl:CD-1(ICR) Mice.	Mouse/CD1 (♂ and ♀) Males: 0, 500, 750ppm. Females: 0, 1000, 1500ppm for 7 days	DAR section B.6.5.3.3 Sulfoxaflor -induced liver effects were consistent with CAR activation resulting in a PB-like MoA; males were more sensitive than females. Neither AhR nor PPARα were involved.	*Geter et al. 2010 (080246)*
MoA Study: Mouse strain suitability.	Mouse/C57Bl/6J WT was suitable alternative to the CD1 mouse.	DAR section B.6.5.3.4 Sulfoxaflor -induced liver effects in C57Bl/6J WT mice were similar to previously observed effects in CD1 mice	*Elcombe 2010*
MoA Study: Mouse/C57Bl/6J WT, Humanised and KO PXR/CAR transgenic models.	No effect with CAR and PXR KO models. Humanised CAR/PXR reacted differently to wildtype.	DAR section B.6.5.3.5 In WT C57BL/6J Sulfoxaflor caused the same liver effects as seen in CD1 mice. In PXR/CAR KO mice, Sulfoxaflor did not induce any liver changes, demonstrating that activation of one or both of these receptors is required to elicit the liver effects seen in WT mice. In PXR/CAR humanised mice slight liver hypertrophic effects occurred but not hepatocellular proliferation. This study demonstrated that Sulfoxaflor, like PB, acts via a CAR-mediated MoA and that mice carrying the human PXR and CAR receptors did not develop hepatocellular proliferation responsible for liver tumour induction. Therefore, Sulfoxaflor -induced rodent liver tumours are not relevant to humans.	*Ross 2010* (100125)

Table 20b: Summary table of mechanistics / Mode of Action (MoA) studies

See next page

Human Relevance Framework for Liver Tumours	Discussion of available data.	DAR section B.6.5.3.6 Sulfoxaflor -induced rodent liver tumours occur via a CAR-mediated MoA for which there is a high level of confidence. There is no evidence of increased hepatocellular proliferation in humanised mice treated with Sulfoxaflor or in humans exposed to high doses of phenobarbital (PB). A hepatocarcinogenic response in rodents for compounds which have data to support a PB-like MoA is considered not relevant to humans. On this basis, the rodent liver tumours associated with administration of high dose levels of Sulfoxaflor would not pose a cancer hazard to humans.	*LeBaron et al., 2010* (100291)
MoA Study: Rat/F344 and Crl:CD(SD) (♂); testosterone elimination and dopamine agonism and / or enhancement MoA study.		DAR section B.6.5.4.1 Support dopamine enhancement MoA for LCT promotion: ↓ Prl levels at 4-wks, ~2-fold dose-dependent ↓ LHR gene expression at 4-wks, ↓ PrlR gene expression at 4-wks.	*Rasoulpour, 2010* (101105)
Proof of Concept Study: Dopamine microdialysis experiment.		DAR section B.6.5.4.2 Sulfoxaflor (400µM and 2mM) produced concentration related increases in the extracellular level of dopamine in the mediobasal hypothalamus. The results indicate that Sulfoxaflor causes a firing dependent increase of dopamine exocytosis from hypothalamic dopaminergic neurones. The data support the hypothesis that through its nAChR partial agonist properties Sulfoxaflor increases dopamine efflux from TIDA neurones in the median eminence, and in turn, this effect is predicted to result in a decrease of prolactin secretion from the pituitary gland in the rat.	*Rowley and Heal* (2011)
MoA Study: Screening for Estrogen Receptor and Androgen Receptor Binding and Transactivation and Aromatase Inhibition.	- hERα AR ligand binding domain - T47D-KBluc cell line (ER) - MDA-kb2 cell line (AR) - Recombinant microsomes	DAR section B.6.5.4.3 Negative for ER binding. Negative for ER and AR transactivation assays (agonism and antagonism). Negative for aromatase (CYP19) inhibition.	*Toole, 2011* (110030)
Human Relevance Framework for Leydig cell Tumours	Discussion of available data.	DAR section B.6.5.4.4 Sulfoxaflor -induced promotion of LCT occurs via a subtle, but chronic, dopamine enhancement MoA in a uniquely susceptible animal model, the Fischer 344 rat. The data for Sulfoxaflor are judged with a moderate degree of confidence to adequately explain the promotion of Fischer rat Leydig cell tumours following chronic dietary administration of Sulfoxaflor, and judged with a very high degree of confidence to support a hormonally-mediated, threshold based, nonlinear MoA.	*Rasoulpour et al., 2011* (110101)

Table 20c: Summary table of mechanistics / Mode of Action (MoA) studies – Preputial Gland

Human Relevance Framework for Preputial Gland Carcinoma	Discussion of available data.	DAR section B.6.5.4.5 The MoA for sulfoxaflor's promotion of preputial gland carcinoma is dopamine enhancement, which is the MoA responsible for the Leydig cell tumour promotion and its associated effects on the epididymides and accessory sex glands of F344/DuCrl rats. This is a hormonally-mediated, threshold based, nonlinear MoA. This MoA is not relevant to humans.	*Stebbins et al. (2011)*

4.10.1 Non-human information

4.10.1.1 Carcinogenicity: oral

Study 1: Rat Combined chronic toxicity/Carcinogenicity (DAR B.6.5.1.1)

Report: Stebbins, K. E., Murray, J. A., Rick, D. L. and Saghir, S. A. (2010). XDE-208: Two-Year Chronic Toxicity/Oncogenicity Study in F344/DuCrl Rats. Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan, 48674 US. Unpublished.

Report No.: DECO HET DR-0404-3134-036. Study ID: 071187.

Dates: 2010

Guidelines: OECD, Guideline 453 (1981): EEC, Part B (1988): USEPA OPPTS 870.4300 (1998): JMAFF, Combined Chronic Toxicity/Oncogenicity Study (2000).

GLP: Yes. This study is fully reliable and satisfies the guideline requirements for a combined chronic toxicity / oncogenicity study in the rat.

Deviations: Yes, at the 4-month time point (03/20/2008), the analytical concentrations and homogeneity values of the high-dose (750 ppm) female diet mix were unacceptable. Female rats were fed this batch for approximately 1 week, a new high dose diet was prepared and confirmed to be analytically acceptable.

Executive Summary:

This study was conducted to assess the potential chronic toxicity and oncogenicity of Sulfoxaflor to male and female F344/DuCrl rats. Groups of 60 male and 60 female F344/DuCrl rats were fed diets formulated to provide 0, 25, 100, 500 (males only) or 750 (females only) ppm Sulfoxaflor for up to two years. The time-weighted average doses, based upon mean feed consumption and body weight data for 24-months were 0, 1.04, 4.24 or 21.3 mg/kg/day for males and 0, 1.28, 5.13 or 39.0 mg/kg/day for females. Ten rats/sex/dose were used for an interim sacrifice and necropsied after one year (chronic toxicity group) and the remaining 50 rats/sex/dose continued to be fed their respective diets for up to two years. After 2 years, there were no statistically-identified differences in mortality for either males or females at any dose level. No treatment related clinical signs were observed due to Sulfoxaflor exposure.

Toxicokinetics: Toxicokinetic analyses of plasma samples at 3 and 12 months showed dose proportionality in systemic dose levels comparable between the two time points. There were no gender differences in plasma concentrations of Sulfoxaflor across the dose levels and times analysed. Urinary elimination of Sulfoxaflor was also dose proportional for both male and female rats at 3, 6 and 12 months, representing 58-127% of the average test material consumed in a 24-hour period.

Effect on bodyweight: Not toxicologically relevant. On Day 729 (study termination), the mean body weight and body weight gain for high dose males were 5.0% and 5.7% lower than controls, respectively. High dose females (750 ppm) showed similar decreases in body weight parameters. On Day 729, the mean body weight and body weight gain for high dose females were 7.5% and 9.1% lower than controls respectively. The body weights of all rats on lower doses were unaffected by treatment with Sulfoxaflor.

Effect on clinical pathology parameters: High-dose males and females had treatment-related and significantly higher cholesterol concentrations compared with concurrent controls at 3, 6 and 12 months, and 3, 6, 12 and 18 months, respectively, with increases ranging from 17.5 to 32.9%. Some minor changes were also observed in some of the haematology parameters and plasma enzymes which are not considered toxicologically significant.

Organ effects; liver: The liver was the primary target organ for histopathological changes in high dose males and females at 12 and 24 months. The absolute and relative liver weights for high-dose males (500 ppm, +17 % and +13% respectively) and females (750 ppm, +3% and +6% respectively) were only increased at 12 months. Non-neoplastic liver effects at 12 and 24 months consisted of hypertrophy of centrilobular and midzonal hepatocytes, necrosis of individual centrilobular hepatocytes, vacuolization (consistent with fatty change) of hepatocytes, and an increase in the severity of aggregates of macrophages/histiocytes. An additional treatment-related liver effect in females given 750 ppm at 24 months consisted of a lower number of basophilic foci of altered hepatocytes. A treatment related neoplastic liver effect at 24 months consisted of a statistically-relevant increase in the incidence of benign hepatocellular adenomas in high dose males. High dose females did not have a treatment-related increase in the incidence of liver tumours. There were no treatment-related liver effects observed microscopically in males or females in the other dose groups.

Organ effects; male reproductive system: At 24 months, males given 100 or 500 ppm had treatment-related, statistically significant increases in absolute and relative testes weights, and treatment-related, statistically significant decreases in absolute and relative epididymal weights. Absolute testes weights were approximately 46% (100 ppm dose) and 62% (500 ppm dose) higher than controls. The higher testicular weights were due to large interstitial (Leydig) cell adenomas in the testes at these dose levels. In addition, high dose males had a treatment-related, statistically significant increase in the incidence of bilateral interstitial cell adenomas of the testes, and a corresponding decrease in the incidence of unilateral interstitial cell tumours, relative to controls. There was also an increase in the incidence of severe bilateral atrophy of seminiferous tubules in the two highest dose groups secondary to malformation by interstitial cell adenomas. At 24 months, the two highest dose groups had treatment-related, statistically significant decreases in absolute and relative epididymal weights in conjunction with a higher incidence of decreased spermatic elements (bilateral, severe) in the lumen of the epididymides of effected males. High dose males had treatment-related, statistically significant increases in the incidence of decreased secretory material in the coagulating glands (severe), prostate (moderate), and seminal vesicles (severe) at 24 months. In addition they also had an increased incidence of carcinoma of the preputial gland.

Although the mode of action for these reproductive organ effects was not investigated as part of this study, the effects are considered to be associated with large interstitial cell adenomas and a disruption in the normal hormonal environment of the hypothalamic, pituitary, gonadal axis. The effects on seminiferous tubules, epididymides, accessory sex glands and preputial gland are likewise considered to be secondary to loss of normal testicular function due to the overwhelming size of the interstitial cell adenomas. It must be stated however that the spontaneous background incidence of Leydig cell tumours is very high in F344/DuCrl rats and is of questionable human relevance. There were no effects observed on the female reproductive system.

Endpoints: Based on treatment-related effects on the testes and epididymides at 100 ppm (4.24 mg/kg bw/day), the no-observed-effect level (NOAEL) for males is 25 ppm (1.04 mg/kg bw/day). However, there is sufficient public domain literature regarding rat Leydig cell tumours and their relevancy to man that this NOAEL for male rats may not be a relevant endpoint for human risk assessment. Ignoring the testicular effects in the male rat, a more appropriate NOAEL can be derived based on decrements in body weight, higher serum cholesterol concentrations, and non-neoplastic liver effects seen in males at the highest dose level tested (500 ppm). A NOAEL (with respect to human relevance) of 100 ppm (4.24 mg/kg bw/day, the next dose down from the highest level tested) is therefore proposed.

Results and Discussion:

Observations:

Dietary analysis

Dose confirmation analyses of all dose levels, plus control and premix, were determined pre-exposure, and at approximate months 4, 8, 12, 18, and 22 of the study. With the exception of the high-dose female (750 ppm) data at 4 months, the concentration of test material in the diet for each dose level ranged from 91.4% to 105.0% of targeted concentrations. With the exception of the high-dose female (750 ppm) homogeneity results at the 4-month analysis, the relative standard deviations (RSD) for all diets sampled ranged from 1.1 % to 6.6% which indicated acceptable homogeneity. The measurements carried out on the high dose female diet at the 4 month time point were unacceptable with mean concentrations of active substance ranging from 65 to 172% of the nominal value. Animals were exposed to this dietary admixture for 1 week before being placed on a new 750 ppm diet which was confirmed to be acceptable (mean Sulfoxaflor concentration of 97.6 % and an RSD of 6.6 %). It is considered that the exposure to the unacceptable diet for a period of only 1 week within the whole study lifetime is of negligible toxicological significance.

Clinical signs of toxicity

There were no clinical findings of significance due to active substance exposure and no dose related responses observed for the lifetime of the study beyond geriatric diseases what would normally be expected from an ageing population. Ophthalmology findings such as incomplete pupillary dilation, pale fundus, cloudy cornea, opaque cornea, opaque lens, microphthalmia or periocular soiling were present but are considered unrelated to treatment with no dose response correlation at any time point. Many of these were considered to be spontaneous, age-related changes comparable in incidence to controls. Detailed clinical observations revealed the inability to evaluate size of pupil (unilateral or bilateral) for a few males or females from all dose levels at various times during the second year of the study. There was no dose response, and consequently this effect is not thought to be treatment

related.

Mortality

After 2 years, there were no statistically significant differences in mortality within the main study groups for either males or females at any dose level (table 6.5.1.1-2). As is typically observed for the F344/DuCrl rat, there was very little mortality for the first 12 – 18 months of the study, after which mortality increased in all dose groups. The distribution of mortalities showed no relationship to treatment. The overall survival rate was higher in females than in males and by the end of the study the total survival rate for all groups was 76%.

Table 4.10.1.1.Study 1.1 (DAR Table 6.5.1.1-2): The incidence of unscheduled euthanasia and survival rate at study end.

Dose mg/kg bw/day	Male				Female				Total mortalities
	0	**1.04**	**4.25**	**21.3**	**0**	**1.28**	**5.13**	**39.0**	
Initial no.	50	50	50	50	50	50	50	50	--
month 0 – 6	0	1	0	0	0	0	0	0	1
month 7 – 12	0	1	0	1	0	0	0	0	2
month 13 – 18	2	1	2	2	0	1	3	2	13
month 19 – 24	14	15	19	14	12	9	10	4	97
total	16	18	21	17	12	10	13	6	113
survival data at termination of study									
total survivors	34	32	29	33	38	40	37	44	367
% survival	68	64	58	66	76	80	74	88	76

Body weight and body weight gain

Body weights and body weight gains (BWG) for males from all treatment groups were comparable to controls throughout the first year of the study. Males given 500 ppm had slight bodyweight reductions of 3 – 5 % relative to controls (treatment-related, statistically significant) from month 17 (Day 512) and continuing to study end at month 24 (Day 729), table 6.5.1.1-3. The body weights of males given 100 or 25 ppm were unaffected by treatment with Sulfoxaflor. BWG was transformed into a body weight loss for males in the final 18 – 24 month interval, ranging from 5 – 10% of final body weight. Females given 750 ppm also had slight bodyweight reductions of 5 – 7 % relative to controls (treatment-related, statistically significant) from early on in the study (less than 6 months) and continuing to study end at month 24 (Day 729), table 6.5.1.1-3. BWG for females in the final 18 – 24 month interval ranged from 6 – 8% of the final body weight. These reductions in BW are marginal, were only observed in the second year of the study and are not considered toxicologically significant.

Food consumption and compound intake

Food consumption by males on all doses varied slightly over the course of the study, typically from 2.4% (day 4 – 8) to a maximum of 7% (day 701 – 708) of the controls. Similarly, female food consumption varied from 2.7% (day 4 – 8) to a maximum of 8% (day 533 – 540)

of the control group. Though these changes were statistically significant they are unlikely to be toxicologically relevant.

Table 4.10.1.1.Study 1.2 (DAR Table 6.5.1.1-3): Selected intervals for body weights and body weight gains for males and females.

Dose mg/kg bw/day	Male				Female			
	0	**1.04**	**4.25**	**21.3**	**0**	**1.28**	**5.13**	**39.0**
Initial wt.	98.1	97.6	96.7	96.4	99.5	99.1	98.6	98.0
month 6	372.8	375.0	369.8	372.0	200.6	199.1	203.6	**193.2***
month 12	432.7	435.4	433.7	429.1	233.1	230.6	237.1	**221.8***
month 18	462.5	468.7	460.4	**450.0***	273.6	269.7	283.1	**260.7***
month 24*	439.3	443.2	418.6	**417.4***	295.4	293.6	305.0	**276.8***
group mean body weight gain (g)								
month 0 - 6	274.6	277.4	273.1	275.6	101.1	100.0	105.0	95.2
month 6 - 12	59.9	60.4	63.9	57.1	32.5	31.5	33.5	28.6
month 12 - 18	29.8	33.3	26.7	20.9	40.5	39.1	46.0	38.9
month 18 - 24	- 23.2	- 25.5	- 41.8	- 32.6	21.8	23.9	21.9	16.1

*This value is the in life body weight data recorded on day 729. Final terminal necropsy body weights reported with organ weights were recorded at a later time point, typically from day 734 to 741.

The actual calculated amount of compound intake after the males received 0, 25, 100 or 500 ppm Sulfoxaflor feed content and the females received 0, 25, 100 or 750 ppm Sulfoxaflor feed content for two years were 0, 1.04, 4.25 or 21.3 mg/kg bw/day for males, and 0, 1.28, 5.13 or 39.0 mg/kg/day for females, respectively.

Clinical pathology:

Haematological findings

The results of selected haematological investigations, including prothrombin times, for 5 sampling time points are summarised in table 6.5.1.1-4. Slight alterations in haematological parameters (RBC, Haemoglobin concentration, and Haematocrit) for males varied from 3 – 5% less than controls (a statistically significant decrease, seen in the 12 month and 18 month time points only). High dose males also had a statistically significant increase in reticulocyte count at 18 months. Males ingesting 4.25 mg/kg bw/day (100 ppm) had a statistically significant decrease in red blood cell count at 18 months. These alterations were interpreted to be unrelated to treatment because of their sporadic occurrence at various time points during the study, and/or because the statistically significant values were within historical control ranges of recently conducted studies from the performing laboratory.

Table 4.10.1.1.Study 1.3 (DAR Table 6.5.1.1-4). Male and Female RBC Count, HGB Concentration, Hematocrit, Reticulocyte Count and Prothrombin time.										
RBC (10^6/µl)	**Males - Dose (mg/kg bw/day)**					**Females - Dose (mg/kg bw/day)**				
	0	**Historic Ctrls**	**1.04**	**4.25**	**21.3**	**0**	**Historic Ctrls**	**1.28**	**5.13**	**39.0**
3 months	9.56±0.25	8.27 – 9.98	9.36±0.28	9.51±0.22	9.40±0.27	8.44±0.24	NA	8.50±0.23	8.36±0.27	8.27±0.15
6 months	9.82±0.47	8.60 – 10.08	9.75±0.19	9.86±0.21	9.96±0.23	8.81±0.17	NA	8.88±0.20	8.65±0.23	8.85±0.21
12 months	9.35±0.26	8.80 – 9.58	9.13±0.26	9.37±0.22	9.13±0.33	7.89±0.87	NA	8.02±0.73	8.07±0.23	8.13±0.45
18 months	9.64±0.28	8.11 – 9.74	9.37±0.29	**9.17*±0.60**	**9.15*±0.34**	8.86±0.16	NA	8.71±0.39	8.61±0.26	8.53±0.17
24 months	8.32±0.45	6.65 – 8.49	7.78±1.50	8.20±0.62	8.19±0.99	7.95±2.39	NA	8.60±0.52	8.34±0.75	7.73±1.45
Hb Conc. (g/dl)										
3 months	16.1±0.5	14.5 – 16.9	15.8±0.2	16.1±0.3	15.9±0.2	15.4±0.4	NA	15.4±0.4	15.1±0.5	15.1±0.3
6 months	16.6±0.8	15.2 – 16.6	16.4±0.3	16.5±0.2	16.7±0.4	16.1±0.3	NA	16.3±0.4	15.8±0.3	16.2±0.5
12 months	15.5±0.4	14.8 – 15.5	15.2±0.4	15.4±0.2	**15.1*±0.4**	14.7±1.7	NA	15.0±0.7	15.0±0.3	14.9±0.8
18 months	16.4±0.4	14.3 – 16.3	16.6±0.6	16.0±1.1	15.8±1.7	16.2±0.3	NA	16.4±0.5	16.1±0.7	15.8±0.5
24 months	14.2±0.5	12.4 – 14.9	13.6±1.9	13.4±1.4	13.5±2.0	14.2±3.6	NA	15.3±0.7	14.9±0.9	13.6±2.7
Haematocrit (%)										
3 months	49.6±1.1	40.2 – 50.0	48.6±0.7	49.3±1.0	48.6±1.1	45.7±1.4	NA	45.8±1.3	45.2±1.3	44.7±1.0
6 months	50.3±2.3	42.7 – 50.8	49.8±0.5	50.5±0.6	50.7±1.2	47.7±1.0	NA	47.8±1.1	46.6±1.1	47.8±1.3
12 months	48.4±1.3	44.0 – 47.6	47.4±1.1	48.3±0.5	**47.0*±1.0**	45.5±5.3	NA	46.8±1.9	46.5±1.1	46.5±2.2
18 months	47.8±1.5	40.4 – 50.8	48.5±1.6	46.9±2.5	46.5±3.5	45.6±0.7	NA	46.2±1.6	45.4±1.5	45.0±1.3
24 months	44.5±1.7	37.7 – 44.9	42.5±5.8	43.0±3.6	43.2±5.2	43.7±10.6	NA	46.7±2.2	46.1±2.6	41.9±7.1
Retic. Count (10^9/l)										
3 months	239.7±42.1	162.6 – 178.2	243.7±89.8	219.8±32.9	239.7±26.4	168.9±25.3	140.3 – 205.8	166.7±21.6	169.1±33.5	169.1±29.7
6 months	203.6±21.9	180.3 – 193.5	206.6±12.4	218.5±36.9	193.6±14.6	181.2±12.7	165.4 – 165.5	187.6±8.4	177.5±21.1	**164.2*±14.2**
12 months	158.7±28.5	150.9 – 156.1	152.3±25.2	159.3±22.1	147.8±32.7	150.3±33.3	159.1 – 166.2	160.1±78.5	154.1±29.2	147.8±25.0
18 months	211.5±30.4	231.6 – 239.5	266.4±66.2	269.0±44.5	**298.4*±83.0**	194.7±9.40	169.7 – 185.9	214.2±32.0	193.8±17.1	201.0±40.0
24 months	230.5±54.2	235.1 – 287.5	293.3±224.8	290.5±55.8	296.9±107.2	201.6±110.8	198.7 – 212.3	152.4±28.4	195.6±100.6	261.4±235.3
Prothrombin time (s)										
3 months	14.9±0.7	NA	14.4±0.7	14.6±0.7	14.6±0.5	13.9±0.3	10.3 – 14.2	13.6±0.4	13.6±0.2	**13.2*±0.4**
6 months	14.5±0.4	NA	14.5±0.4	14.5±0.9	14.7±0.6	13.7±0.4	11.3 – 14.3	13.6±0.5	13.4±0.3	**13.0*±0.3**
12 months	15.4±0.4	NA	15.4±0.5	15.6±0.7	15.4±0.8	14.1±0.4	11.6 – 13.5	14.0±0.5	14.4±0.5	13.6 ±0.7
18 months	15.0±0.4	NA	15.4±0.4	15.6±0.9	15.0±0.5	14.4±0.6	11.2 – 14.5	14.6±0.5	14.6±0.5	14.0 ±0.3
24 months	15.4±0.6	NA	15.3±1.4	15.9±0.6	15.3±0.6	15.2±1.0	11.7 – 14.6	15.3±0.6	14.7±0.4	**14.3*±0.8**

Historical control values taken from 2 – 8 studies between 2005 and 2009; * Statistically different from control mean by Dunnett's Test, alpha = 0.05; NA: not available in original study report or company summaries. Values are means ± 1 standard deviation.

The reticulocyte count was increased in all dose groups at 18 and 24 months, with 41% and 28% increases respectively, at the highest dose (21.3 mg/kg bw/day). Since a corroborating decrease in RBC count and an increase in MCV (2%) were only slight at 18 months, with little change from controls at 24 months, the increased reticulocyte count is considered a non-adverse finding. Alterations in haematological parameters for females are also presented in table 6.5.1.1-4. Females in the high dose group (39.0 mg/kg bw/day) had a statistically significant decrease in reticulocyte count at 6 months, and statistically significant shorter prothrombin times at 3, 6 and 24 months. These alterations appear to be unrelated to treatment because the values were within or near historical control ranges and they are not consistent or repeated at the other time points of the study.

Clinical Biochemistry

High dose males (21.3 mg/kg bw/day) had treatment-related, statistically significant increases in cholesterol concentrations at 3, 6 and 12 months, and high dose females (39.0 mg/kg bw/day) had treatment-related, statistically significant increases cholesterol concentrations at 3, 6, 12, and 18 months (table 6.5.1.1-5). Females on the mid dose of 5.13 mg/kg bw/day (100 ppm) had a slight but significant increase in cholesterol concentration at 3 months. However, this seems unrelated to treatment because of the lack of repeatability and consistency at later time points of the study. This conclusion is also supported by a lack of an effect on cholesterol at this dose level in the previously conducted 90-day rat study.

Males and females from the high-dose groups (21.3 mg/kg bw/day and 39.0 mg/kg bw/day, respectively) had significant decreases in liver enzyme activities for ALT, ALP, and/or AST at various time points during the study (table 6.5.1.1-5). Similarly for mid dose males (4.25 mg/kg bw/day), there were significant decreases in ALP activity at 3 and 6 months, and a lower AST activity at 3 and 12 months. Mid dose females (5.13 mg/kg bw/day) also had a slight but significant decrease in AST activity at 3 months. Low dose males (1.04 mg/kg bw/day) had a significant decrease in ALP activity at 6 months, and low dose females (1.28 mg/kg bw/day) had a significant decrease in ALP activity at 3 months. There were no statistically significant alterations in liver enzyme activities at 24 months. All of the lower liver enzyme activities are not considered to be toxicologically relevant because of the lack of a clear dose-response and inconsistent occurrence during the study time points. The absence of significant liver enzyme activity effects in the previously conducted 90-day rat study supports the results presented here and also indicates a lack of toxicological significance with respect to liver enzyme activities in plasma.

Urinalysis

High dose males had a significant increase in urine volume and lower urine specific gravity at 6 months (4.3 ± 1.4 vs. 2.5 ± 0.6 ml in concurrent controls). Males on all doses had a significant increase in urine volume at 12 months relative to controls with little to no effect present at 3, 18 or 24 months. There were no significant increases or decreases in female urinary output or changes in urine density. All male urine volume results were within the ranges of historical controls. There were no histopathological effects in the urinary tract of high dose males at the 12-month interim and 24-month sacrifices. The variations in urine volume and density are not considered treatment related or toxicologically relevant.

There was an absence of detectable bilirubin in the urine of mid dose males (4.25 mg/kg bw/day) at 6 and 12 months, high dose males at 3, 6, 12 and 18 months, and high dose females at 3, 6, and 12 months. At 24 months, most of the males and females from the

Table 4.10.1.1.Study 1.5 (DAR Table 6.5.1.1-5.) **Selected Male and Female Clinical Chemistry Results.**										
CHOL (mg/dl)	**Males - Dose (mg/kg bw/day)**					**Females - Dose (mg/kg bw/day)**				
	0	**Historic Ctrls**	**1.04**	**4.25**	**21.3**	**0**	**Historic Ctrls**	**1.28**	**5.13**	**39.0**
3 months	59±3	47 - 62	64±6	62±9	**77*±6**	93±7	80 – 94	101±11	**103*±10**	**118*±6**
6 months	72±8	56 - 78	78±7	74±12	**85*±9**	116±9	92 – 109	112±11	121±11	**138*±12**
12 months	79±9	76 – 100	**95*±11**	89±9	**105*±16**	143±23	117 – 131	137±12	140±11	**168*±13**
18 months	111±18	113 – 151	131±27	116±14	127±37	133±14	114 – 133	134±17	124±10	**158*±16**
24 months	159±40	160 – 201	173±108	141±34	157±63	152±33	117 – 152	140±20	139±19	157±42
ALT (U/L)										
3 months	67±9	54 - 83	64±8	55±7	59±15	46±9	42 – 89	44±8	42±6	41±3
6 months	85±18	83 – 101	87±16	76±16	**68*±11**	71±30	59 – 90	73±30	67±18	56±9
12 months	107±18	101 – 131	130±36	92±13	100±21	73±19	71 – 77	76±18	65±9	59±12
18 months	63±12	65 – 83	70±26	75±14	**48$±9**	56±10	59 – 72	54±11	70±36	44±8
24 months	49±8	49 – 122	63±31	57±20	64±23	88±122	47 – 83	49±10	49±6	46±15
ALP (U/L)										
3 months	109±12	94 – 118	100±5	**99*±7**	**96*±9**	83±11	71 – 88	**71*±6**	75±6	**66*±8**
6 months	103±11	94 – 107	**92*±7**	**90*±9**	94±7	74±8	61 – 85	73±10	70±8	**61*±10**
12 months	95±9	95 – 112	87±7	84±10	86±12	58±7	48 – 81	59±12	59±13	**46*±6**
18 months	77±16	69 – 110	82±23	82±15	69±13	54±9	50 – 58	50±10	76±53	**43$±5**
24 months	76±16	70 – 145	76±37	86±26	88±39	77±71	56 – 87	51±8	45±7	58±37
AST (U/L)										
3 months	128±22	99 – 134	128±19	**103*±17**	106±25	96±9	95 – 127	93±7	**86*±7**	**83*±7**
6 months	160±42	139 – 156	161±43	151±33	**119*±17**	123±26	96 – 155	137±66	123±29	115±23
12 months	135±17	117 – 144	141±25	**115*±16**	**110*±13**	115±26	108 – 154	131±30	108±21	105±30
18 months	94±14	108 – 156	104±33	172±206	**75$±10**	119±26	108 – 117	96±25	119±45	**76*±17**
24 months	81±12	86 - 319	112±54	106±53	115±54	346±820	85 - 218	85±23	89±18	89±41

Historical control values taken from 4 – 8 studies between 2005 and 2009; * Statistically different from control mean by Dunnett's Test, alpha = 0.05; $ Statistically different from control mean by Wilcoxon's Test, alpha = 0.05; NA: not available in original study report. Values are means ± 1 standard deviation. CHOL = cholesterol; ALT = alanine aminotransferase; ALP = alkaline phosphatase; AST = aspartate aminotransferase.

control group and all treatment groups had no detectable bilirubin in the urine. When bilirubin was detectable there was no apparent dose effect and rarely was the level described as more than slight. In general, the absence of bilirubin in the urine is a non-adverse finding and the results do not indicate a reason for a toxicological concern. Significant toxicological effects on the liver would be associated with a dramatic increase (rather than a decrease) in the amount of bilirubin in the urine. This is not observed. Furthermore, perturbations in kidney function are not supported because there is no corroboration with rises in blood urea nitrogen, plasma alkaline phosphatase (ALP) and plasma γ-glutamyltransferase (γGT), nor was there any increase in creatinine.

Plasma and Urine Toxicokinetics

Concentrations of Sulfoxaflor in plasma were used as an indicator of systemic dose and for the assessment of dose proportionality. Mean concentrations of plasma Sulfoxaflor taken at the 3 month time point was compared to the 12 month time point. Both male and female rats displayed a linear increased proportionality in sulfoxalor plasma concentration (and thus systemic dose) with increasing test material intake.

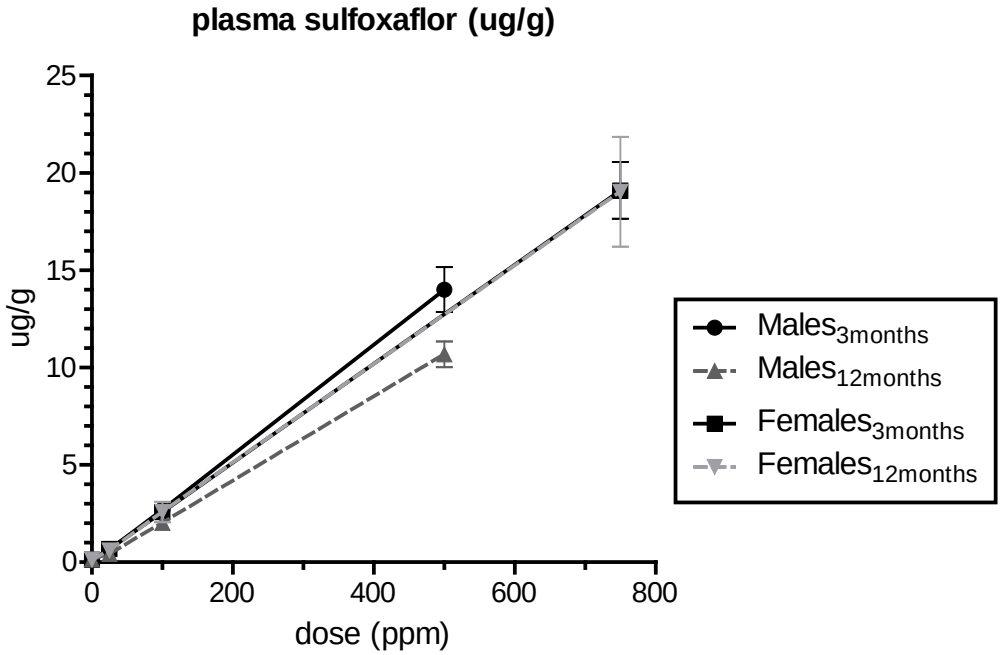


Figure 4.10.1.1.Study 1.1 (DAR Figure 6.5.1.1-1): mean plasma concentrations of Sulfoxaflor in samples taken on day 94 (month 3) at 09.00 and on day 352 (month 12) at 09.00 from both male and female rats. The data represents the mean of 5 animals and error bars represent ± 1 sd. Data taken from original study report.

The 12-month plasma $AUC_{24\ hours}$ levels increased proportionally with increased Sulfoxaflor dietary intake as indicated by mean values of 9.7, 42.1 and 228 µgh·mL $^{-1}$ for male rats fed on 25, 100 and 500 ppm diets. Likewise, the 12-month plasma $AUC_{24\ hours}$ levels in female rats increased proportionally with increased Sulfoxaflor dietary intake, as indicated by mean values of 12.7, 50.8 and 422 µg·h·mL^{-1} for animals fed on 25, 100 and 750 ppm diets.

The equivalence in dose-corrected $AUC_{24\ hour}$ values and their equivalence between males and females indicate (1) there is no saturation of systemic absorption, (2) there is no saturation of

systemic elimination, and (3) there is gender-equivalence in the kinetics of dietary administered Sulfoxaflor. The mean dose-corrected $AUC_{24\ hour}$ values were 10.4, 11.1, and 12.0 $\mu g \cdot h \cdot mL^{-1}$ for male rats fed on 25, 100 and 500 ppm diets. The mean dose-corrected $AUC_{24\ hour}$ values were 10.0, 9.96, and 11.8 $\mu gh \cdot mL^{-1}$ for female rats fed on 25, 100 and 750 ppm diets.

Mean plasma elimination half-lives ranged from 11-14 hours in male rats and from 9-10 hours in female rats (100 and 750 ppm dose levels) and confirms that elimination of systemic Sulfoxaflor is not saturated (within the confines of the doses tested) with increasing dose.

Urinary elimination of Sulfoxaflor is also dose proportional for both male and female rats. At 3 months, male rats eliminated average amounts of Sulfoxaflor (across doses) ranging from 58 – 85% of the test material consumed over 24 hours. At 6 months, average percent urinary elimination in male rats ranged from 79 – 99%; and at 12 months ranged from 62 – 68%. At 3 months, female rats eliminated average amounts of Sulfoxaflor (across doses) ranging from 98 - 127% of the test material consumed over 24 hours. At 6 months, average percent urinary elimination in female rats ranged from 102 - 105%; and at 12 months ranged from 69 - 78%.

Sacrifice and Pathology:

Organ weights

12 month interim sacrifice

A number of statistically significant differences in absolute and relative organ weights were recorded. These differences are not considered to be toxicologically significant if considered alone; there is no dose response and no obvious pattern. High dose males had significant increases in absolute (+17%) and relative liver weights (+13%) over concurrent controls but a dose response is not evident from the lower doses (table 6.5.1.1-6). High dose females had slightly higher absolute (+3%) and relative liver weights (+6%, statistically significant) compared with concurrent controls but not historical controls. The liver weight increases for both sexes coincided with hypertrophy of centrilobular and midzonal hepatocytes.

Females given 100 (5.13 mg/kg bw/day) or 750 ppm (39.0 mg/kg bw/day) had statistically-identified higher absolute and relative ovary weights – increased by 19 and 21%; and 16 and 23% respectively (table 6.5.1.1-6). The higher ovary weights showed no clear dose response and were very similar in magnitude to the upper level of historical control ovary weights from studies recently conducted at the same performing laboratory. There were no corresponding histopathological effects in the ovaries at any dose level. The higher ovary weights were interpreted to be unrelated to treatment. Males given 25 ppm (1.04 mg/kg bw/day) Sulfoxaflor had statistically-identified lower relative testicular and epididymal weights. There were no differences associated with the 2 higher dosing regimes.

24 month scheduled sacrifice

A number of statistically significant differences in absolute and relative organ weights were also recorded at the end of the oncogenicity study. Most of these differences are not considered to be toxicologically significant; except in the case for mid to high dose males where testicular and epididymal weights showed clear treatment responses. There was no toxicologically relevant effect on overall liver weights. Absolute testes weights of males given 100 (4.25 mg/kg bw/day) or 500 ppm (21.3mg/kg bw/day) were approximately 46%

and 62% higher than controls, respectively. The higher testes weights were due to the presence of large interstitial (Leydig) cell tumours. The lower epididymal weights (reduced by 23 – 26%) were associated with decreased spermatic elements (bilateral, severe) in the lumen of the epididymides of males from the mid and high dose groups.

Table 4.10.1.1.Study 1.5 (DAR Table 6.5.1.1-6.) Selected Organ Weights – 12 Month interim sacrifice

Males: Dose (ppm)	**Historical Control**	**0**	**25**	**100**	**500**
Final Body Weight (g)	421.4 - 435.8	391.5±22.1	416.5±33.6	410.3±21.9	404.0±13.8
Liver, absolute (g)	10.617 - 10.987	9.428±0.658	10.185±1.163	10.009±0.502	**11.035*±0.907**
Liver, rel. (g/100g bw)	2.438 - 2.606	2.409±0.111	2.440±0.108	2.441±0.079	**2.728*±0.151**
Testes, absolute (g)	NA	3.479±0.516	3.197±0.326	3.422±0.218	3.344±0.128
Testes, rel. (g/100g bw)	NA	0.891±0.144	**0.769±0.076$**	0.834±0.026	0.828±0.034
Epidid., absolute (g)	NA	0.986±0.090	0.930±0.068	1.012±0.051	0.958±0.051
Epidid., rel. (g/100g bw)	NA	0.252±0.017	**0.224±0.019***	0.247±0.013	0.237±0.012
Females: Dose (ppm)	**Historical Control**	**0**	**25**	**100**	**750**
Final Body Weight (g)	217.9 - 224.3	214.2±10.0	215.8±13.4	216.3±13.7	207.9±11.2
Liver, absolute (g)	5.354 - 6.755	5.591±0.323	5.495±0.384	5.690±0.468	5.772±0.262
Liver, rel. (g/100g bw)	2.451 - 3.119	2.615±0.175	2.547±0.067	2.628±0.098	**2.779*±0.091**
Ovaries, absolute (g)	0.051 - 0.075	0.063±0.012	0.075±0.014	**0.076*±0.007**	**0.078*±0.010**
Ovaries, rel. (g/100g bw)	0.023 - 0.035	0.030±0.006	0.035±0.006	**0.035*±0.004**	**0.037*±0.004**

Historical control values taken from 4 studies between 2005 and 2009; * Statistically different from control mean by Dunnett's Test, alpha = 0.05; $ Statistically different from control mean by Wilcoxon's Test, alpha = 0.05; NA: not available in original study report or company summaries. Values are means ± 1 standard deviation.

There was a clear link between animals with large interstitial cell adenomas (and concomitant severe atrophy of testicular seminiferous tubules) and the presence of decreased amounts of sperm in the epididymides, presumably secondary to the testicular perturbations.

Females from the high dose group only had a slight (but significant) 7.5% decrease in final body weight, relative to controls. Most of the observed changes in female organ weights at the high dose appear to be related to the significant decrease in overall mean body weight at this dose level. There is no clear dose response unlike the case with the male genitalia/accessory organ weights presented earlier. High dose females had statistically significant increases in relative kidney, liver and brain weights, and a statistically significant decrease in absolute heart weight. There was no effect on ovary weight at this time point.

Gross pathology:

12 month interim sacrifice.

There were no treatment-related gross pathological effects at any dose level.

24 month scheduled sacrifice.

There were no treatment-related gross pathologic effects at any dose level.

Table 4.10.1.1.Study 1.6 (DAR Table 6.5.1.1-7.) Selected Organ Weights – 24 Month scheduled sacrifice					
Males: Dose (ppm)	**Historical Control**	**0**	**25**	**100**	**500**
Final Body Weight (g)	390.2 - 416.4	415.2±46.7	418.4±35.6	396.0±34.7	394.2±29.6
Liver, absolute (g)	NA	12.146±4.161	11.856±2.352	12.056±2.386	12.019±1.694
Liver, rel. (g/100g bw)	2.819 - 3.288	2.987±1.308	2.864±0.697	3.084±0.808	**3.083$^{\$}$±0.692**
Testes, absolute (g)	4.053 - 4.933	3.720±1.686	3.933±1.451	**5.423*±2.139**	**6.025*±2.146**
Testes, rel. (g/100g bw)	0.972 - 1.254	0.906±0.421	0.940±0.337	**1.359*±0.510**	**1.519*±0.521**
Epidid., absolute (g)	0.505-0.601	0.560±0.172	0.488±0.111	**0.432*±0.126**	**0.413*±0.129**
Epidid., rel. (g/100g bw)	0.127-0.144	0.135±0.040	0.116±0.025	**0.110*±0.033**	**0.105*±0.033**
Females: Dose (ppm)	**Historical Control**	**0**	**25**	**100**	**750**
Final Body Weight (g)	272.0 - 279.5	278.2±22.8	275.4±19.9	283.4±27.7	**257.2*±23.1**
Heart, absolute (g)	0.803 - 0.914	0.904±0.084	0.885±0.050	0.895±0.074	**0.862*±0.068**
Heart, rel. (g/100g bw)	NA	0.327±0.046	0.323±0.031	0.317±0.027	0.337±0.033
Kidneys, absolute (g)	NA	1.920±0.695	1.895±0.154	1.948±0.152	1.898±0.153
Kidneys, rel. (g/100g bw)	0.704 - 0.740	0.695±0.087	0.690±0.062	0.691±0.062	**0.743*±0.081**
Liver, absolute (g)	NA	7.587±1.228	7.330±0.830	7.788±1.301	7.674±1.348
Liver, rel. (g/100g bw)	2.656-2.768	2.752±0.627	2.662±0.243	2.749±0.386	**2.990$^{\$}$±0.475**
Ovaries, absolute (g)	NA	0.073±0.035	0.064±0.016	0.070±0.019	0.067±0.021
Ovaries, rel. (g/100g bw)	NA	0.026±0.013	0.023±0.006	0.025±0.007	0.026±0.008

Historical control values taken from 4 studies between 2005 and 2009; * Statistically different from control mean by Dunnett's Test, alpha = 0.05; $ Statistically different from control mean by Wilcoxon's Test, alpha = 0.05; NA: not available in original study report or company summaries. Values are means ± 1 standard deviation.

Non-neoplastic histopathology:

12 month interim sacrifice

The liver was the target organ for histopathological effects in high dose males and females (table 6.5.1.1-8), with males being more consistently and severely affected at these dose levels. There were no treatment-related effects observed in the livers of males or females in the low and mid dose groups.

All high dose male rats had very slight, slight or moderate hepatocellular hypertrophy, with altered tinctorial properties (increased cytoplasmic eosinophilia), involving the centrilobular to midzonal regions of the hepatic lobule. The majority of these males also had very slight or slight multifocal individual cell necrosis of centrilobular hepatocytes, and slight vacuolisation of hepatocytes, consistent with fatty change. In addition, males with the greatest degree of hepatocellular hypertrophy, necrosis, and vacuolisation, had slight multifocal aggregates of macrophages – histiocytes. These cells were likely associated with increased phagocytic

activity that is normally required to remove cellular debris (such as necrotic elements originating from individual hepatocyte necrosis). There were no neoplastic or pre-neoplastic effects observed in the liver at the 12 month interim sacrifice.

In high dose females, 8/10 rats had very slight centrilobular to midzonal hypertrophy of hepatocytes, 3/10 had very slight necrosis of individual centrilobular hepatocytes, and 4/10 had slight multifocal vacuolisation of hepatocytes, consistent with fatty change.

The microscopic changes were present in all three lobes of the liver examined in male and female rats; however, they were more readily apparent in the right lateral lobe. Histopathological changes in the liver were consistent with the increased liver weights and probably also with the increased serum cholesterol noted previously in high dose males and females. Many of the histopathological changes at the 12 month interim sacrifice would appear to be spontaneous alterations, though the highest administered dose of Sulfoxaflor tends to push these histopathological changes into a slightly more severe category, especially in males.

Table 4.10.1.1.Study 1.7 (DAR Table 6.5.1.1-8) Incidence (number of animals) of selected histopathological liver effects – 12 month interim sacrifice (n = 10, all doses)

		Males				Females			
Dose (ppm)		**0**	**25**	**100**	**500**	**0**	**25**	**100**	**750**
Aggregates of macrophages histiocytes; multifocal, -	very slight	9	10	10	5	9	9	10	9
	slight	0	0	0	**5**	0	0	0	0
Hypertrophy; with altered tinctorial properties; hepatocyte; centrilobular/midzonal, -	very slight	0	0	0	**2**	0	0	0	**8**
	slight	0	0	0	**6**	0	0	0	0
	moderate	0	0	0	**2**	0	0	0	0
Necrosis; individual cell; hepatocyte; centrilobular; multifocal, -	very slight	1	0	0	**7**	0	0	0	**3**
	slight	0	0	0	1	0	0	0	0
Vacuolisation; consistent with fatty change; hepatocyte; multifocal, -	very slight	6	10	9	2	4	2	6	5
	slight	0	0	0	**7**	0	0	0	**4**

Values in **Bold type** indicate effects judged to be treatment related

24 month scheduled sacrifice.

Liver lesions: The liver was also the primary target organ at the 24 month terminal sacrifice for histopathological effects in high dose males and females (table 6.5.1.1-9). There were no treatment-related effects observed microscopically in the livers of males or females on the low and mid dose regimes.

In high dose males and females, the majority of animals had very slight or slight hepatocellular hypertrophy, with altered tinctorial properties, involving the centrilobular to midzonal regions of the hepatic lobule and is considered treatment related. Similarly, high dose animals also had a statistically significant, increased incidence of very slight multifocal individual cell necrosis of centrilobular hepatocytes, and very slight multifocal aggregates of macrophages – histiocytes – possibly involved in removing cellular debris (individual cell

Table 4.10.1.1.Study 1.8 (DAR Table 6.5.1.1-9) Incidence (number of animals) of selected histopathological liver effects – 24 month terminal sacrifice (n = 50, all doses)									
		Males				Females			
Dose (ppm)		**0**	**25**	**100**	**500**	**0**	**25**	**100**	**750**
Aggregates of Macrophages, histiocytes; multifocal, -	very slight	32	40	26	27	38	38	34	26*
	slight	4	1	4	**16***	4	0	8	**21***
Focus of Cellular Alteration; hepatocyte; basophilic.	6 – 10	22	14	14	23	7	3	7	**18***
	11 – 20	5	12	11	1	19	21	18	18
	≥ 21	0	1	1	0	19	18	18	**2***
Hypertrophy; with altered tinctorial properties; hepatocyte; centrilobular/midzonal, -	very slight	2	0	0	0	0	1	0	**33***
	slight	0	0	0	**34***	0	0	0	**5**
	moderate	0	0	0	0	0	0	0	0
Necrosis; individual cell; hepatocyte; centrilobular; multifocal, -	very slight	2	0	0	**24***	0	0	1	**22***
	slight	0	0	0	1	0	0	0	1
Vacuolisation; consistent with fatty change; hepatocyte; multifocal, -	very slight	21	25	21	23	27	35	36	9*
	slight	17	8	12	20	9	7	5	**28***
	moderate	1	1	2	0	0	0	1	**7***

Values in **Bold type** indicate effects judged to be treatment related; *Statistically significant by Yate's Chi-Square test, alpha = 0.05.

Table 4.10.1.1.Study 1.9 (DAR Table 6.5.1.1-10) Incidence (number of animals) of selected male reproductive histopathology – 24 month terminal sacrifice (n = 50, all doses), * p < 0.05					
Dose (ppm)	**severity**	**0**	**25**	**100**	**500**
TESTES					
atrophy; seminiferous tubule; bilateral	severe	13	15	**25***	**34***
EPIDIDYMIDES					
decreased spermatic elements; bilateral	severe	21	23	29	**37***
COAGULATING GLAND					
decreased secretory material	slight	11	9	11	6
	moderate	14	21	15	17
	severe	10	11	16	**21***
PROSTATE					
decreased secretory material	slight	22	22	24	12
	moderate	13	15	17	**25***
SEMINAL VESICLE					
decreased secretory material	slight	11	9	12	6
	moderate	14	21	14	18
	severe	10	11	16	**21***

hepatocyte necrosis). Additional treatment-related liver effects in high dose females consisted of a statistically significant increased incidence of slight or moderate multifocal vacuolisation of hepatocytes (consistent with fatty change), and a statistically significant decreased incidence of rats with the highest number of basophilic foci of altered hepatocytes (quantified as 21 or more basophilic foci in the three standard liver sections examined microscopically).

Male reproductive lesions: Treatment-related testicular lesions were also recorded and consisted of severe bilateral atrophy of seminiferous tubules in males at the mid and high doses (100, 500ppm). At the end of the study there were lower absolute and relative epididymal weights, along with a higher incidence of decreased spermatic elements (bilateral, severe) in the lumen of the epididymides of these males. High dose males exhibited treatment-related statistically significant increases in the incidence of decreased secretory material in the coagulating glands (severe), prostate (moderate), and seminal vesicles (severe), see table 6.5.1.1-10.

Neoplastic changes:

12 month interim sacrifice

Liver: There were no neoplastic or pre-neoplastic effects observed in the liver at the 12 month interim sacrifice.

Testes: Males from the mid and high dose groups each had 3/10 animals with a small (only visible microscopically), unilateral benign, Leydig cell adenoma of the testes at 12 months, versus 0/10 males from the control group and 1/10 males from the 25 ppm (1.04 mg/kg bw/day) group. The historical control incidence of testicular Leydig (interstitial) cell adenomas in males sacrificed at 12 months ranged from 0/10 to 3/10 in the six previously conducted chronic toxicity/oncogenicity studies from the performing laboratory. Because the incidences of Leydig cell adenomas from the 100 and 500 ppm groups were within the historical control range, and there was no dose response despite the known dose-proportionality of systemic blood levels and $AUC_{24\ hour}$ of Sulfoxaflor (see toxicokinetics section), this finding may be interpreted as being unrelated to treatment at the 12 month time point.

24 month scheduled sacrifice.

Dietary administration of Sulfoxaflor resulted in tumours of the liver, testes and the preputial glands in male rats. There was no evidence of carcinogenicity in female rats. The statistical analyses of the tumours in male rats were based upon Fisher's Exact Test for pair-wise comparisons and the Exact Test for trend.

Liver: Statistically significant trends ($p<0.01$) were seen for both hepatocellular adenomas and the combined (adenomas/carcinomas). When compared to controls, a statistically significant increase in pairwise comparison was seen for hepatocellular adenomas ($p<0.01$) and combined adenomas/carcinomas ($p<0.05$, driven by the adenoma response) at the highest dose (500 ppm, 21.3mg/kg bw/d). The incidences of liver tumours at the high dose (33%) exceeded the testing laboratories historical control range of 2 – 12% for the adenomas or 2 – 14% for the combined liver tumours (table 6.5.1.1-11).

Testes: As shown in table 6.5.1.1-12, there was a significant trend for the Leydig cell adenomas and a pair-wise significance ($p<0.01$) at the high dose (500 ppm) when compared

to controls for the bilateral neoplasm, but not for the unilateral neoplasm. This is treatment related even if the incidences of the combined adenomas (92%) were similar to the testing laboratory's historical control mean value (85%) and lay within its range (76 – 92%). F344 rats are known to have high background rates for Leydig cell tumours but the incidences observed for the bilateral tumour in conjunction with increased testicular mass and negative effects on the histology of secondary reproductive tissues indicate a treatment related effect (increased tumour load) by Sulfoxaflor in the mid and high dose regimes.

Table 4.10.1.1.Study 1.10 (DAR Table 6.5.1.1-11) Sulfoxaflor - F344/DuCrl Male Liver Tumour Rates

Liver Lesion	**0 ppm (0 mg/kg/day)**	**25 ppm (1.04 mg/kg/day)**	**100 ppm (4.24 mg/kg/day)**	**500 ppm (21.3 mg/kg/day)**
Adenomas	4/50	2/48	5/50	16/49
(%)	(8)	(4)	(10)	(33)
p =	0.00002**	0.88829	0.50000	0.00213**
Carcinomas	3/50	1/48	1/50	0/49
(%)	(6)	(2)	(2)	(0)
p =	0.05383	0.93625	0.94127	1.00000
Combined	7/50	3/48	6/50	16/49
(%)	(14)	(6)	(12)	(33)
p =	0.00043**	0.94744	0.72322	0.02440*
$adenomas (%)	6			
range (%)	2 – 12			
$carcinomas (%)	0.5			
range (%)	0 – 2			
$combined (%)	6.5			
range (%)	2 – 14			

* p < 0.05, ** p <0.01; $ historical control data, 4 studies from 2005 – 2009

Table 4.10.1.1.Study 1.11 (DAR Table 6.5.1.1-12) Sulfoxaflor - F344/DuCrl Testicular Leydig Cell (Interstitial Cell) Tumour Rates

Testicular Lesion	0 ppm (0 mg/kg/day)	25 ppm (1.04 mg/kg/day)	100 ppm (4.24 mg/kg/day)	500 ppm (21.3 mg/kg/day)
Adenomas (unilateral)	12/50	8/50	5/50	2/50
(%)	(24)	(16)	(10)	(4)
p =	0.0025**[N]	0.8947	0.9845	0.9996
Adenomas (bilateral)	32/50	38/48	40/50	44/49
(%)	(64)	(76)	(80)	(88)
p =	0.0065**	0.1376	0.0591	0.0046**
Combined	44/50	46/48	45/50	46/49
(%)	(88)	(92)	(90)	(92)
p =	0.3495	0.3703	0.5000	0.3703
[$]adenomas; unilateral	14			
(%)	12 – 16			
range (%)				
[$]adenomas; bilateral	71			
(%)	64 – 76			
range (%)				
[$]combined (%)	85			
range (%)	76 – 92			

* p < 0.05, ** p <0.01; $ historical control data, 4 studies from 2005 – 2009

Preputial gland: The preputial gland was not a protocol-required tissue for histopathological examination, but the gross observations of masses and/or nodules in this tissue made it necessary to examine the affected glands microscopically. Preputial glands are found in both rats and mice and are paired, modified sebaceous glands located in the inguinal region adjacent to the penis and vagina respectively. Spontaneous adenomas are very rare but they can be induced by several compounds such as 3-monochloro-propane-1,2-diol (3-MCPD, α-chlorohydrin, a rodenticide with an unrelated mode of action to Sulfoxaflor, which incidentally also gives rise to increased incidences of Leydig cell tumours in rats – JECFA toxicology monograph, FAS 48-JECFA 57/401). There appears to be an increased incidence of carcinoma of the preputial gland (clitoral glands in females) at the high dose in males only (table 6.5.1.1-13). However, histopathological examination of the preputial gland was conducted only when the presence of a gross lesion such as a mass or nodule was observed upon macroscopic examination of the urogenital area containing this gland. This means that not all of the animals (50 animals/group) underwent histopathological examination of the preputial gland. It also means that no meaningful statistical analysis of the tumour incidence can be conducted. Therefore, there is an absence of data with respect to the effect of treatment, i.e. the true association of the dose of Sulfoxaflor and occurrence of preputial gland tumours is unknown. Also, sex-hormone analyses were not conducted in the current study so it is not possible to draw any conclusions with regards to alterations in the endocrine balance of the treated animals. Historical control data for the incidences of carcinoma of the preputial gland amongst 4 previously conducted carcinogenicity studies in the same performing laboratory was also presented but is of limited value for the same reasons as given above.

Table 4.10.1.1.Study 1.12 (DAR Table 6.5.1.1-13) Sulfoxaflor - F344/DuCrl Preputial Tumours/Lesions in male rats

Preputial Lesion	0 ppm (0 mg/kg/day)	25 ppm (1.04 mg/kg/day)	100 ppm (4.24 mg/kg/day)	500 ppm (21.3 mg/kg/day)
mass / nodule / abscess* (%)	8/50 (16)	8/50 (16)	7/50 (14)	10/50 (20)
carcinoma** (%)	5/8 (63)	7/8 (88)	7/7 (100)	10/10 (100)
$carcinoma (%)	1/11 (9)	2/10 (20)	0/4 (0)	6/6 (100)

* number of animals examined for gross pathology of preputial gland due to presence of an unknown mass; ** number of animals for which preputial glands were examined and found positive for carcinoma; 2 females from the low dose group also had visible lesions of the preputial gland, these 2 females had their preputial glands histologically evaluated and they were found to have tumours; $ historical control data from 4 separate studies, no details given, number of animals examined and found positive for carcinoma of the preputial gland (note, true incidence unknown, total number of animals per study not stated, all animals not examined for histological evidence of carcinoma).

Conclusions

The mortality rate did not differ significantly between control and treated groups. There were no clinical signs observed after Sulfoxaflor exposure. The body weights of males and females exposed to the low and mid doses of Sulfoxaflor were unaffected by treatment. Statistically significant reductions in body weight were recorded in both sexes at the highest dose but are not toxicologically significant (body weight decrease < 10%, i.e. high dose males exhibited body weight reductions of 5% while females exhibited just over 6% at study termination). A similar situation was observed with reductions in body weight gains of 5.7 – 9.1% for males and females respectively at study termination.

Haematological findings were generally unremarkable. The reticulocyte count was increased in all male rat dose groups at 18 and 24 months, with 41% and 28% increases respectively at the highest dose. This was not considered adverse because there was no corroborating evidence from other parameters such as a significant decrease in RBC count and an increase in MCV. Similarly, clinical biochemistry was unremarkable with respect to plasma enzymes but high dose males had treatment-related, statistically significant increases in cholesterol concentrations at 3, 6 and 12 months, and high dose females had treatment-related, statistically significant increases in cholesterol concentrations at 3, 6, 12, and 18 months – these increases ranged from 17.5 to 32.9%. Urinalysis results were unremarkable, even with statistically significant differences between treated and controls in male animals. All male urine volume results were within the ranges of historical controls and there were no histopathological effects in the urinary tract of high dose males at the 12-month interim and 24-month sacrifices. There was no evidence for perturbations in either liver or kidney function amongst both sexes.

Both male and female rats displayed a linear increased proportionality in sulfoxalor plasma concentration (and thus systemic dose) with increasing test material intake. The equivalence in dose-corrected $AUC_{24\ hour}$ values and their equivalence between males and females indicate

(1) there is no saturation of systemic absorption, (2) there is no saturation of systemic elimination, and (3) there is gender-equivalence in the kinetics of dietary administered Sulfoxaflor. Urinary elimination of Sulfoxaflor is also dose proportional for both male and female rats.

A number of statistically significant differences in absolute and relative organ weights were recorded at the end of the oncogenicity study. Most of these differences are not considered to be toxicologically significant; except in the case for mid to high dose males where testicular and epididymal weights showed clear treatment responses and are associated with significant pathology (absolute testes weights were approximately 46% and 62% higher than controls for the mid and high dose groups, respectively and similarly for absolute epididymal weights which were reduced by 23% and 26%, respectively). The higher testes weights were due to the presence of interstitial (Leydig) cell adenomas in the testes. There was a clear link between animals with higher testicular weight and severe atrophy of seminiferous tubules, decreased amounts of sperm in the epididymides, and decreased secretory material in the coagulating glands, prostate, and seminal vesicles; all presumably secondary to the testicular adenomas.

The liver was the primary target organ for histopathological effects in high dose males and females at both 12 and 24 months while the reproductive organs of males in the mid (100 ppm) and high dose (500 ppm) groups was also a primary target at the end of the study. The absolute and relative liver weights were only increased at 12 months, in the range of 3.2 to 17%. Non-neoplastic liver effects at 12 and 24 months consisted of hypertrophy of centrilobular and midzonal hepatocytes, necrosis of individual centrilobular hepatocytes, vacuolisation (females only) consistent with fatty change of hepatocytes, and an increase in the severity of aggregates of macrophages/histiocytes in both sexes exposed to the highest dose of Sulfoxaflor. An additional treatment-related liver effect in high dose females at 24 months consisted of a lower number of basophilic foci of altered hepatocytes.

A treatment-related, statistically significant increase in the incidence of hepatocellular adenomas was seen in males in the high dose group. High dose females did not have a treatment-related increase in the incidence of liver tumours. There were no treatment-related liver effects in lower dose males or females. Further studies (dual CAR/PXR knockout mice, gene expression with real-time PCR and Ki-67 immunohistochemical staining, liver enzyme analysis and molecular markers of PB-like activity and nuclear receptor analysis, described later) and a formal framework analysis investigating the proposed mode of action (MoA) support a threshold based, mitogenic response similar to a phenobarbital (PB) like MoA for these liver tumours. These tumours are considered to be rodent specific with little relevance to human hazard assessment.

The incidence of Leydig cell adenomas in at least one testis (unilateral Leydig cell adenomas) from all dose groups was comparable to or less than controls at 24 months. Notwithstanding the high background incidence of Leydig cell tumours in F344 rats, high dose males had a statistically significant increase in the incidence of bilateral Leydig cell adenomas of the testes, and a corresponding decrease in the incidence of unilateral Leydig cell tumours, relative to controls. This is interpreted as a distinct treatment response and assumes that an increase in the incidence of bilateral adenomas over controls is indicative of an increased tumour load with increasing Sulfoxaflor exposure. Indirectly this is borne out with the observed increase in testicular weight associated with increasing Sulfoxaflor dose.

High dose males had statistically significant increases in the incidence of decreased secretory

material in the coagulating glands (severe), prostate (moderate), and seminal vesicles (severe) at 24 months. All the effects described thus far are interpreted to be the result of Leydig cell adenomas, and the effects on seminiferous tubules, epididymides, accessory sex glands are considered secondary to loss of normal testicular function due to the size of the Leydig cell adenomas. Although the MoA for the effects on the male reproductive organs was not investigated as part of this study, MoA data for the Leydig cell adenomas was submitted separately. It should also be noted that rat Leydig cell adenomas have questionable significance with respect to human risk assessment. This will be discussed in later sections.

High dose males also have an apparent increased incidence of carcinoma of the preputial gland but there is insufficient data to confirm the true magnitude of this incidence and if the figures recorded (number of histopathological positives relative to number of animals with macroscopic palatable preputial gland masses) are actually correct or reflect an underestimate of the true incidence of this tumour. It is possible for instance, that if all the animals in a given dose group were evaluated histologically for the presence of preputial tumours, then, this incidence may rise because of the recognition of early changes in the tissue that reflect neoplastic progression. In the case of the preputial gland carcinomas, possible alterations in the endocrine balance of these rats may account for the carcinomas but the MoA remains largely unknown. It should also be noted that humans do not have an anatomical correlate to the preputial gland of rodents and therefore, the higher incidence of preputial gland carcinomas in Fischer 344 rats may have no relevance to humans.

Based on treatment-related effects on the testes, seminiferous tubules and epididymides at 4.24 mg/kg bw/day (100ppm) and higher, the no observed adverse effect level (NOAEL) for males is 1.04 mg/kg bw/day (25ppm). The NOAEL for females is 5.13mg/kg bw/day (100ppm), based on raised serum cholesterol concentrations, and histopathological liver effects at 39 mg/kg bw/day (750ppm), the highest dose level tested.

The pathways for the regulation of the hypothalamo-pituitary-testis (HPT) of rats and humans are similar, such that chemical mediated reductions in testosterone and oestradiol or effects on their receptors that diminish molecular recognition will increase LH blood levels. Hence, compounds that induce LCTs in rats by disruption of the HPT axis may also pose a risk to human health. There is an exception though, amongst several hormonal modes of action through which such compounds operate, two are generally considered not relevant for humans – Gonadotropin Releasing Hormone (GnRH) agonism and dopamine agonism. Some of the mechanistic studies described later try to elucidate the mechanism by which Sulfoxaflor induces LCTs.

If we consider that rat LCTs have little to no relevance for humans then a NOAEL based on such effects is overly conservative with respect to human risk assessment. In this case, a revised NOAEL of 4.24 mg/kg bw/day (100ppm) is proposed on the basis of increased serum cholesterol concentrations, and histopathological liver effects in high dose males (21.3mg/kg bw/day, 500ppm).

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Study 2: **Carcinogenicity study in mice (DAR B.6.5.2.1)**

Report: Thomas, J., Marshall, V. A., Yano, B. L. and Rick, D., (2010b). XDE-208: Oncogenicity Study in Crl:CD1(ICR) Mice. Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan, 48674. Unpublished.

Report No.: DECO HET DR-0404-3134-060. Study ID: 081102.

Dates: 2010

Guidelines: OECD, Guideline 451 (1981): EEC, Part B.32 (1988): US EPA OPPTS 870.4200 (1998): JMAFF, Oncogenicity Study (2000).

GLP: Yes. This study is fully reliable and satisfies the guideline requirements for an oncogenicity study in the mouse.

Deviations: Haematological investigations consisted of white blood cell differential counts only.

Executive Summary:

In this carcinogenicity study with Crl:CD1(ICR) mice, Sulfoxaflor was administered in the diet to groups of 50 animals per dose at 0, 25, 100, or 750 ppm for males and at 0, 25, 250 or 1250 ppm to females for 18 months. These concentrations corresponded to time-weighted average doses of 0, 2.54, 10.4 or 79.6 mg/kg/day for males, and 0, 3.43, 33.9, or 176 mg/kg/day for females, respectively.

Toxicokinetics: Toxicokinetic analyses of plasma samples at 3 and 12 months showed dose proportionality with respect to systemic dose with no gender differences. Urinary elimination of Sulfoxaflor was similarly dose proportional for both male and female mice.

Effect on bodyweight and clinical pathology parameters: Not toxicologically relevant. There were no treatment-related changes in clinical observations, body weights and body weight gains, feed consumption, ophthalmologic observations, or total and differential WBC counts

in any of the Sulfoxaflor treated groups.

Organ effects; liver: The liver was the primary target organ. The absolute and relative liver weights of high dose males (750ppm, 79.6 mg/kg bw/day) were increased 87 and 79% respectively. Males were more susceptible than females; in addition, adverse effects were noted at lower exposure levels, at necropsy, there was a treatment-related increase in the incidence of mass nodules and multifocal pale foci. Hepatocellular adenomas and/or carcinomas were present in 60% of high dose male mice but in only 10% of high dose female mice (1250ppm, 176 mg/kg bw/day). Treatment-related non-neoplastic liver effects consisted of increases in the incidences of eosinophilic and vacuolated foci of cellular alteration in high dose males; slight to moderate centrilobular/midzonal or panlobular hepatocellular hypertrophy with altered tinctorial properties (increased cytoplasmic eosinophilia) consistent with liver enzyme induction in high dose males and females; very slight or slight multifocal individual cell necrosis of hepatocytes in high dose males and females (very slight); very slight fatty change in centrilobular/midzonal hepatocytes in high dose males and females and increased incidence of hepatocytes in mitosis in high dose males.

Organ effects; dermal inflammation: High dose males had an exacerbation in the cumulative incidence of spontaneous dermatitis which is common in CD-1 mice. Histologically, these were characterized by subacute to chronic inflammation, variable epidermal ulceration and acanthosis. Associated with the ulcerative dermatitis in high dose males was an increased incidence of reactive plasmacytosis of the local submandibular lymph nodes. In high dose females there was a non-statistically significant increase in lymphosarcoma.

Mode of Action: Mode of Action (MoA) investigations into liver tumours were conducted separately and are reported later in this section of the DAR. A phenobarbital (PB) like MoA is been postulated for sulfoxyflor induced rodent liver tumours.

Endpoints: Based on treatment-related adverse effects on the liver at 750 ppm (79.6 mg/kg bw/day, LOAEL), which included massively increased liver weights, increased incidence of liver nodules, liver hypertrophy, liver histopathology (necrosis, fatty change), and increased incidence of liver tumours, the no-observed-adverse-effect level (NOAEL) for males is proposed to be 100 ppm (10.4 mg/kg bw/day).

Results and Discussion:

Observations:

Dietary analysis

Dose confirmation analyses of all dose levels, plus control and premix, were determined pre-exposure, and at approximate months 4, 8, 12, and 16 of the study. Analyses of all Sulfoxaflor test diets indicated the mean concentration for each dose level ranged from 95.4 to 99.0% of targeted concentrations. The homogeneity of Sulfoxaflor in diets was determined for the low dose male and high dose female diets with relative standard deviations between 1.4 and 5.4%.

Clinical signs of toxicity

There were no clinical findings of significance due to active substance exposure and no dose related responses observed for the lifetime of the study beyond geriatric diseases what would normally be expected from an ageing population. Sporadic incidences of clinical observations

noted across all dose-groups including controls were present but are considered unrelated to treatment with no dose response correlation at any time point. Many of these were considered to be spontaneous, age-related changes comparable in incidence to controls. Dermatitis was noted across all dose groups but not in a dose-related manner. Ophthalmic observations on the day prior to study termination consisted of sporadic incidences of several pathologies such as pale fundus, cloudy cornea, opaque cornea, cloudy lens, and opaque lens. These observations however, were not considered to be treatment related.

Mortality

After 18 months there were no statistically significant differences in mortality between the study groups for either males or females at any dose level (table 6.5.2.1-2). The mortality rates at the end of the study were 12, 28, 14, and 26% for males in the control, 25, 100, and 750 ppm groups (0, 2.54, 10.4, 79.6 mg/kg bw/day), respectively; and 26, 28, 12, and 30% for females in the control, 25, 250, and 1250 ppm groups (0, 3.43, 33.9, 176 mg/kg bw/day), respectively. There was very little mortality for the first 6 – 12 months of the study, after which mortality increased in all dose groups. The distribution of mortalities showed no relationship to treatment. The cause of death or moribundity in a small proportion of high dose males (6 of 50) was attributed to treatment-related hepatocellular carcinoma or adenoma with or without other co-existing conditions such as ulcerative dermatitis or ascites. The overall survival rate was similar in both sexes and by the end of the study the total survival rate for all groups was 78%.

Body weight and body weight gain

There were no treatment-related or statistically significant differences in body weight throughout the duration of the study. Body weight gains relative to Day 1 were also not affected by treatment throughout the duration of the study in Sulfoxaflor treated males and females across all dose groups. Body weights and body weight gains (BWG) for males and females from all treatment groups were comparable to controls, table 6.5.2.1-3. BWG was transformed into a slight body weight loss for males in the final 12 – 18 month interval, ranging from 0.6 – 5% of final body weight. Females continued to gain slightly in body weight up to the end of the study. BWG for females in the final 12 – 28 month interval ranged from 2.3 – 4.4% of the final body weight. Changes in BW observed in this study are marginal and are not considered toxicologically significant.

Table 4.10.1.1.Study 2.1 (DAR Table 6.5.2.1-2): The incidence of unscheduled euthanasia and survival rate at study end.

Dose mg/kg bw/day	Male				Female				Total mortalities
	0	**2.54**	**10.4**	**79.6**	**0**	**3.43**	**33.9**	**176**	
Initial no.	50	50	50	50	50	50	50	50	--
month 0 – 6	0	1	1	0	**1**	0	1	0	4
month 7 – 12	0	3	0	0	3	4	1	5	16
month 13 – 18	6	10	6	13	9	10	4	10	68
total	6	14	7	13	13	14	6	15	88
survival data at termination of study									
total survivors	44	36	43	37	37	36	44	35	312
% survival	88	72	86	74	74	72	88	70	78

Table 4.10.1.1.Study 2.2 (DAR Table 6.5.2.1-3): Selected intervals for body weights and body weight gains for males and females.

Dose mg/kg bw/day	Male				Female			
	0	**2.54**	**10.4**	**79.6**	**0**	**3.43**	**33.9**	**176**
Initial wt.	31.2	31.0	31.2	31.2	23.7	23.1	22.9	23.0
month 6	47.5	49.9	47.6	46.8	35.0	34.9	36.0	34.7
month 12	49.9	51.1	49.6	48.9	38.1	39.4	40.2	38.3
month 18	47.6	50.8	48.6	48.3	39.0	41.2	41.5	39.6
group mean body weight gain (g)								
month 0 - 6	16.4	18.9	16.4	15.6	11.3	11.8	13.2	11.7
month 6 - 12	2.4	1.2	2.0	2.1	3.1	4.5	4.2	3.6
month 12 - 18	- 2.3	- 0.3	- 1.0	- 0.6	0.9	1.8	1.3	1.3

Food consumption and compound intake

Overall there were no treatment-related differences in feed consumption throughout the duration of the study; average feed consumption data for each group was very similar to controls with one minor exception. Feed consumption was increased and statistically significant for females during the interval represented by test days 365 – 372 for the 250 and 1250 ppm groups. This was considered unrelated to treatment as it represents a sporadic result at this one interval from the whole study (i.e. 4.7g and 4.8g for the mid and high dose respectively vs 4.4g for controls).

The actual calculated amount of compound intake after males received 0, 25, 100 or 750 ppm Sulfoxaflor feed and females received 0, 25, 250 or 1250 ppm Sulfoxaflor feed for 18 months were on average 0, 2.54, 10.4 or 79.6 mg/kg bw/day for males, and 0, 3.43, 33.9 or 176 mg/kg bw/day for females, respectively.

Clinical pathology

Haematological findings:

There were no treatment-related or statistically significant changes in the total white blood cell counts in males and females. Differential white blood cell percentages did not reveal any treatment-related changes for either sex. There were no red blood cell parameters measured, no clinical chemistry and no urinalysis.

Plasma and Urine Toxicokinetics:

Concentrations of Sulfoxaflor in plasma were used as an indicator of systemic dose and for the assessment of dose proportionality. Mean concentrations of plasma Sulfoxaflor taken at the 3 month time point was compared to the 12 month time point. Both male and female rats

displayed a linear increased proportionality in sulfoxalor plasma concentration (and thus systemic dose) with increasing test material intake.

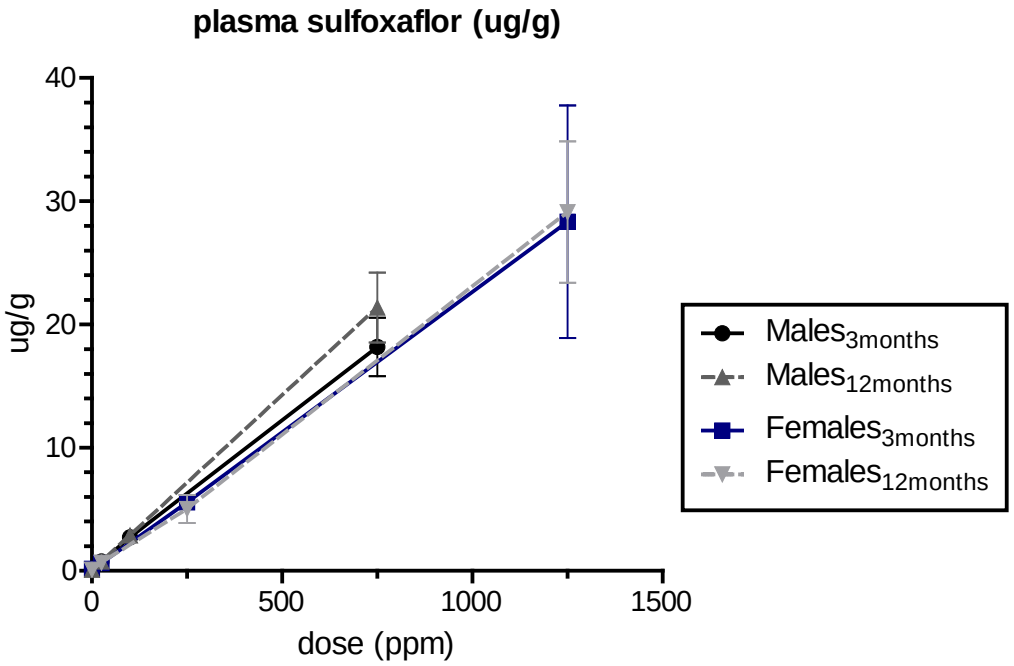

Figure 4.10.1.1.Study 2.1 (DAR Figure 6.5.2.1-1): mean plasma concentrations of Sulfoxaflor in samples taken at month 3 and at month 12 from both male and female rats. The data represents the mean of 5 animals and error bars represent ± 1 sd. Data taken from individual animal data in the original study report.

The mean plasma concentration of Sulfoxaflor in mice after 3 months of dietary administration corresponding to test material intakes of 2.8, 10.6, and 89.6 mg/kg/day (male); and 3.7, 45.2, and 197 mg/kg/day (female) were 0.7, 2.7, and 18.2 µg/g; and 0.6, 5.6, and 28.3 µg/g in male and female mice respectively.

The 12-month test material intake values were 2.2, 8.3, and 64.8 mg/kg/day for male mice; and 3.0, 30.7, and 144 mg/kg/day for female mice. Although these dietary intake levels decreased slightly from 3 to 12 months, the plasma concentrations of Sulfoxaflor at 3 and 12 months were quite similar. The mean plasma concentrations of Sulfoxaflor in mice after 12 months of dietary administration were 0.8, 2.9, and 21.4 µg/g in males; and 0.7, 5.0, and 29.1 µg/g in female mice.

Statistical analysis of the concentration of parent compound in urine indicated that there was no deviation from linearity for urinary excretion of the test material, in either sex.

Sacrifice and Pathology

Organ weights:

There were no treatment-related or statistically significant differences in terminal body weights of males and females treated with Sulfoxaflor when compared to their respective controls.

A number of statistically significant differences in absolute and relative organ weights were

recorded. The mean absolute and relative liver weights of high dose males were increased 87 and 79% respectively; similarly, in high dose females they were increased 51 and 47%, respectively when compared to their respective controls (table 6.5.2.1-4). These increases were statistically significant and considered treatment related as they corresponded to the treatment-related hepatocellular hypertrophy and increased incidences of hepatocellular neoplasia at these dose levels.

The mean absolute liver weight and relative liver weight of males given 100 ppm was approximately 19% higher than those of controls, however, these weight differences were not statistically significant. These higher weights were driven by several individual mice in this group (6/43 with liver weights in excess of 4g) and one animal in particular (#2563) was excluded because it had an extreme value for liver weight (>32g) – caused by a large diffusely cystic liver virtually 10-times the normal size (not an ademona or carcinoma); others were also increased (4.2 – 9.6g) due to the presence of mass nodules. There were no clear treatment-related microscopic changes in the livers of males given 100 ppm but the higher incidences in liver weights at this dose (6/43) and the highest dose (7/37, all > 6g, 6.5 – 16g) were considered related to treatment. There were no treatment-related increases in liver weights of males given 25ppm (1/36 with a liver weight > 3.8g) or females given 25 or 250ppm (except for 1 animal, #2702, excluded with liver weight > 27g).

Several other organs exhibited similar but sporadic large increases in organ weight (ovarian and uterine weights, all doses). Ovarian weight data for animal #2740 (absolute weight > 18g) in the 250ppm group was excluded because it also had an extreme value that would skew the mean making it nonsensical if it was included in table 6.5.2.1-4. The mean absolute and relative adrenal gland weights of females given 1250 ppm were slightly higher and statistically significant when compared to their respective controls (but still within the performing laboratory's recent historical control range). This finding was considered unrelated to treatment due to the absence of any associated clinical signs and histopathological findings. The mean relative brain weight and absolute epididymal weight of males given 25 ppm were marginally lower or higher, respectively and statistically significant, as compared to their respective controls. These were considered unrelated to treatment due to their isolated nature and lack of a dose-response relationship. Unlike the rats from the combined carcinogenicity / chronic toxicity study, testicular weight was unaffected by treatment and there was no evidence for Leydig cell tumours.

Table 4.10.1.1.Study 2.3 (DAR Table 6.5.2.1-4). Selected Organ Weights – Mouse 18 Month scheduled sacrifice

Males: Dose (ppm)	**Historical Control**	**0**	**25**	**100**	**750**
Final Body Weight (g)	43.7 - 45.7	46.9±6.0	50.0±6.1	47.9±7.2	48.0±5.0
Liver, absolute (g)	2.411 -2.444	2.537±0.792	2.476±0.410	3.034±1.637 **(1)**	**4.754$^{\$}$±3.256**
Liver, rel. (g/100g bw)	5.284 -5.585	5.441±1.616	4.987±0.793	6.466±3.448 **(1)**	**9.747$^{\$}$±6.384**
Brain, absolute (g)	NA	0.533±0.027	0.523±0.027	0.526±0.037	0.520±0.029
Brain, rel. (g/100g bw)	1.185 -1.196	1.155±0.157	**1.062*±0.150**	1.120±0.164	1.092±0.122
Testes, absolute (g)	NA	0.249±0.046	0.252±0.035	0.241±0.039	0.263±0.035
Testes, rel. (g/100g bw)	NA	0.535±0.087	0.510±0.085	0.514±0.100	0.551±0.083
Epidid., absolute (g)	0.120 - 0.146	0.122±0.015	**0.134*±0.023**	0.127±0.019	0.124±0.016
Epidid., rel. (g/100g bw)	NA	0.262±0.033	0.269±0.051	0.269±0.049	0.258±0.032
Females: Dose (ppm)	**Historical Control**	**0**	**25**	**250**	**1250**
Final Body Weight (g)	36.8 -39.1	39.0±6.0	41.9±8.9	41.8±7.0	40.1±5.1
Liver, absolute (g)	2.007 – 2.145	1.887±0.420	2.018±0.687 **(2)**	2.072±0.515	**2.858$^{\$}$±0.807**
Liver, rel. (g/100g bw)	5.351 -5.748	4.882±1.026	4.897±1.282 **(2)**	5.015±1.168	**7.205$^{\$}$±2.210**
Adrenals, absolute (g)	0.0116 - 0.0153	0.010±0.002	0.0105±0.0030	0.0106±0.0026	**0.0125*±0.003**
Adrenals, rel. (g/100g bw)	0.0312 - 0.395	0.027±0.007	0.0258±0.0081	0.0259±0.0072	**0.0318*±0.010**
Ovaries, absolute (g)	NA	0.064±0.147	0.052±0.125	0.102±0.203 **(3)**	0.065±0.098
Ovaries, rel. (g/100g bw)	NA	0.161±0.364	0.130±0.353	0.232±0.443 **(3)**	0.162±0.246

Historical control values taken from 3 studies between 2005 and 2007; * Statistically different from control mean by Dunnett's Test, alpha = 0.05; $^{\$}$ Statistically different from control mean by Wilcoxon's Test, alpha = 0.05; NA: not available in original study report or company summaries. Values are means ± 1 standard deviation. (1) Liver weight data for #2563 in the 100ppm group excluded, values are too extreme (liver abs > 32g, liver rel > 46g); (2) Liver weight data for #2702 in the 25ppm group excluded, values are too extreme and give a falsely inflated mean value and sd (liver abs > 27g, liver rel. > 42g); (3) Ovarian weight data for #2740 in the 250ppm group excluded, values too extreme (absolute wt > 18g, rel. wt > 34g). The high values are still due to some other animals in this group with much heavier ovarian weights, though not as extreme as for #2740.

Gross pathology:

Treatment related gross observations are summarised in table 6.5.2.1-5. The number of mice with one or more mass nodules on the liver, indicative of possible neoplasia was more than

double in high dose males when compared to the incidence within the 0 dose controls. High dose females also had a higher incidence of one or more mass nodules in the liver when compared to their concurrent controls. These changes in the livers of high-dose males and females were considered treatment-related. In addition, the incidence of multifocal, pale foci (suggestive of multiple foci of pre-neoplastic or neoplastic change) was greatest in high dose

Table 4.10.1.1.Study 2.4 (DAR Table 6.5.2.1-5): Incidence (number of animals) of treatment related observations

	Males				Females			
Dose (ppm)	**0**	**25**	**100**	**750**	**0**	**25**	**250**	**1250**
Liver:	50	50	50	50	49	50	50	50
animals with≥ 1 mass nodules	11	8	12	**25**	1	1	3	**7**
foci, pale and multifocal	0	0	0	**6**	0	3	2	0
Skin & Subcutaneous tissue:	50	24	16	50	50	18	9	50
inflammation (all sites)	8	10	11	**16(?)**	7	4	2	8
inflammation (neck)	1	2	4	**10(?)**	0	0	0	0

Values in **Bold type** (may?) indicate effects judged to be treatment related.

males when compared to controls and was also interpreted to be related to treatment. There were no treatment-related gross findings in the livers of lower dose males and females.

Sporadic incidences of skin inflammation (dermatitis) were noted in all dose groups including controls. Dermatitis affected different regions of the body such as on the ear pinnae, neck, head, eyelid, back and forelimbs. The incidence of dermatitis, regardless of its location was greatest in high dose males compared to the controls. This was particularly evident for the incidence of dermatitis on the neck region. Dermatitis in CD-1 mice is a common background spontaneous lesion, noted particularly in males. The exact etiology for the spontaneous dermatitis is unknown. It typically starts in the ear pinna resulting in necrosis of the ears in some mice. In some, it progresses and involves the neck and shoulders. Dermatitis affecting one or more sites was observed in some mice across all treated groups including controls. The higher incidence of dermatitis in high dose males (and to a much lesser extent in high dose females), may be interpreted as an exacerbation of an already spontaneous background lesion. However, it is difficult to say what contribution if any, is made by exposure to Sulfoxaflor. The only real comparison that can be made is the controls *vs.* the high dose group, the raw data was recorded from fewer animals in the interim doses and this prevents a true incidence from being calculated for the low and mid dose groups only. This prevents any recognition of a dose response (assuming proportionality of the response is not maintained). In addition, there was no evidence of dermal erythema or lymphocyte proliferation in the auricular lymph nodes of treated CBA/J mice from the LLNA skin sensitisation study (B6.2.6.1), however it is recognised that different strains of mouse display differential sensitivity to the occurrence of idiopathic dermal inflammation.

There were no other gross findings with significant deviations from controls as a consequence of treatment. All other gross findings where noted, were considered spontaneous or age-related changes non-associated with exposure to Sulfoxaflor due to their (1) low incidence; (2) sporadic occurrence; (3) lack of dose-response and / or (4) inability to calculate incidence in the low to mid dose groups.

Non-neoplastic histopathology:

The liver was the target organ for histopathological effects in high dose males and females (table 6.5.2.1-6), with high dose males (750ppm; 79.6mg/kg bw/day) being more severely affected even though they received less test article than the high dose females (1250ppm;

176mg/kg bw/day). There were no significant treatment-related effects observed in the livers of animals from the lower dose groups.

Liver lesions: Treatment-related changes in the liver consisted of increases in the incidences of eosinophilic and vacuolated foci of hepatocellular alteration in males given 750 ppm. Males and females given 750 ppm or 1250 ppm had treatment related, slight to moderate hypertrophy of hepatocytes in the centrilobular/midzonal region or in the entire lobule (panlobular). The affected hepatocytes had a very slight alteration in the tinctorial properties characterized by a homogeneous cytoplasm with increased eosinophilia, consistent with possible increase in smooth endoplasmic reticulum and induction of P450 enzymes. Other treatment-related changes in the liver consisted of multifocal individual cell necrosis of hepatocytes in males given 750 ppm (very slight or slight) and in females given 1250 ppm (very slight). This was characterized by the presence of scattered, individual necrotic hepatocytes containing eosinophilic cytoplasm with or without karyorrhexis and frequently surrounded and/or infiltrated with small numbers of neutrophils. A very slight treatment-related vacuolisation consistent with fatty change was noted in the centrilobular/midzonal hepatocytes characterised by the presence of fine round lipid vacuoles in the cytoplasm of high dose males and females, respectively. A very slight or slight treatment-related increase in the numbers of hepatocytes in mitosis was also noted in some males receiving 750 ppm of test article in the diet.

Table 4.10.1.1.Study 2.5 (DAR Table 6.5.2.1-6): Incidence (number of animals) of selected non-neoplastic histopathological liver effects (n = 50[1] livers examined, all doses)

		Males				Females			
Dose (ppm)		**0**	**25**	**100**	**750**	**0[1]**	**25**	**250**	**1250**
Focus of Cellular Alteration; eosinophilic; 1-5		3	2	3	**10**	0	0	0	0
Focus of Cellular Alteration; vacuolated (combined)		1	0	1	**6**	0	0	0	0
Hypertrophy; with altered tinctorial properties; hepatocyte; centrilobular/midzonal, -	very slight	6	8	7	0	1	0	5	4
	slight	0	0	4	**13***	1	0	9	**13***
	moderate	0	0	0	**22***	0	0	0	**12***
Hypertrophy; with altered tinctorial properties; hepatocyte; panlobular, -	very slight	--	--	--	--	--	--	--	--
	slight	0	0	0	**10***	3	2	4	**12***
	moderate	0	0	0	**2**	0	0	0	0
Necrosis; individual cell; hepatocyte; multifocal, -	very slight	9	4	3	**26***	1	3	1	**6**
	slight	0	0	0	**2**	0	0	0	0
Vacuolisation; consistent with fatty change; hepatocyte; centrilobular/midzonal, -	very slight	2	1	1	**16***	1	0	0	**5**
	slight	3	5	3	1	0	0	0	0
	moderate	0	0	1	0	0	0	0	0
Mitotic alteration; increased; hepatocyte, -	very slight	5	1	1	**8**	1	3	2	0
	slight	0	0	0	**2**	0	0	0	0

Values in **Bold type** indicate effects judged to be treatment related; *Statistically significant by Yate's Chi-Square test, alpha = 0.05. – not recorded. [1] Note: control female #2623 died by crush trauma in week 3, the final number of animals in the female control group should be 49 and not 50.

The only treatment-related non-neoplastic change in the livers of the female mid-range dose group (250ppm) was a very slight to slight centrilobular/midzonal hepatocellular hypertrophy with altered tinctorial cytoplasmic changes in 14 out of 50 females (compared with 2 out of 50 for controls and 17 out of 50 high dose females). The hepatocellular hypertrophy in the females at this level is not considered adverse because it is present in isolation with no association with other treatment-related neoplastic changes such as increased numbers of mitotic hepatocytes, hepatocyte necrosis or other degenerative changes, and a lack of statistically significant or treatment-related increase in liver weights.

Other lesions including inflammation: Skin samples from suspected sites of dermatitis were characterized by variable severities of subacute to chronic inflammation of the dermis, acanthosis of the epidermis (epidermal hyperplasia) and ulceration. Associated with the increased incidence of ulcerative dermatitis particularly in the neck region of high dose males, was an increase in the incidence of plasmacytosis (increased numbers of plasma cells) of the medullary cords in the local submandibular lymph nodes (possibly an immune response to secondary bacterial infection through the ulcerated skin), table 6.5.2.1-7. The increased incidence of dermatitis in high dose males would appear to be treatment-related but the high spontaneous incidence of this lesion in CD-1 mice make interpretation difficult as does the fact that lower numbers of animals were examined for these effects in the low to mid dose

groups making any comments on the true incidence or dose response open to a lot of uncertainty. There would appear to be no treatment-related increase in the incidences of dermatitis in females at any dose and in males at any dose less than the highest dose group.

Table 4.10.1.1.Study 2.6 (DAR Table 6.5.2.1-7) Incidence (number of animals) of selected non-neoplastic histopathological lesions (variable numbers of animals examined across doses)

		Males				Females			
Dose (ppm)		**0**	**25**	**100**	**750**	**0[1]**	**25**	**250**	**1250**
Skin & subcutaneous (n)		*50*	*24*	*16*	*50*	*49*	*18*	*9*	*50*
Inflammation:									
neck		1	2	4	10	0	0	0	0
all types, any severity	any	8	10	11	16	4	2	0	1
ulceration, neck, focal	severity	0	2	3	6	1	1	0	1
ulceration (all types)	any severity	7	8	9	11	4	1	2	7
Acanthosis									
epidermis, neck	moderate	0	1	2	**8**	1	1	0	1
Multiple organs (n)		*2*	*1*	*2*	*0*	*4*	*6*	*0*	*5*
Lymphosarcoma; -		1	1	1	0	2	1	0	5
Lymph node: Submandib (n)		*10*	*5*	*8*	*16*	*4*	*2*	*0*	*11*
Plasmacytosis	any severity	10	5	7	16	4	2	0	7

Values in **Bold type** (may) indicate effects judged to be treatment related. [1]Note: control female #2623 died by crush trauma in week 3 of the study. Any pathological observations for this animal are inconsequential due to the brief exposure period relative to other animals on the study, thus the final number of animals in the female control group should be 49 and not 50.

In the original study report, there was a non-statistically significant increase in lymphosarcoma in the female high dose group, however this is not interpreted here to be toxicologically significant from the raw data because of the low number of animals examined. There is no evidence of a treatment related effect. For example, it is reported 5/5 are positive at the high dose, there is no data for the 250ppm female group, and 2/4 from the control group. Does this imply that there is a 50% incidence in the control group? This is highly unlikely.

Male reproductive lesions: In contrast to the F344 rat, there were no treatment-related testicular lesions or effects on other secondary reproductive structures at any dose level.

Neoplastic changes:

Dietary administration of Sulfoxaflor resulted in increased tumours of the liver in a dose dependent manner. Male mice were more susceptible to the development of adenomas and carcinomas than female mice. In contrast to the F344 rat, there were no tumours of the testes or the preputial/clitoral glands. There was no evidence of Leydig cell hyperplasia in male mice.

Liver: As shown in tables 6.5.2.1-8a and 6.5.2.1-8b, treatment-related neoplastic effects

consisted of statistically significant ($p < 0.01$) increases in the incidences of hepatocellular adenomas and carcinomas in high dose males and females when compared to their respective controls. Male mice were much more sensitive to the neoplastic effects of Sulfoxaflor – 60% of the high dose males developed hepatocellular adenomas and/or carcinomas (*vs.* 26% in male controls), as opposed to 11% of the high dose females (*vs.* 2% in female controls). Statistical analyses were based upon Fisher's Exact Test for pair-wise comparisons and the Exact Test for trend.

Table 4.10.1.1.Study 2.7 (DAR Table 6.5.2.1-8a): Sulfoxaflor – CD-1 Mice: Male Liver Tumour Rates

Liver Lesion	**0 ppm (0 mg/kg/day)**	**25 ppm (2.54 mg/kg/day)**	**100 ppm (10.4 mg/kg/day)**	**750 ppm (79.6 mg/kg/day)**
*total animals**	*50*	*46*	*49*	*50*
Adenomas	12/50	6/46	10/49	**24*/50**
(%)	(24)	(13)	(20)	**(48)**
p =	0.000	0.955	0.749	**0.011**
Carcinomas	2/50	0/46	4/49	**17*/50**
(%)	(4)	(0)	(8)	**(34)**
p =	0.000	1.000	0.329	**0.000**
Combined	13/50	6/46	12/49	**30/49**
(%)	(26)	(13)	(24)	**(60)**
p =	0.000	0.972	0.657	**< 0.001**
animals with both an adenoma and a carcinoma:	1	0	2	11
$adenomas (%)	13			
range (%)	10 – 18			
$carcinomas (%)	0.6			
range (%)	0 – 2			
$combined (%)	14			
range (%)	10 – 20			

*Tumour rates are based on number of tumour bearing animals relative to number of animals examined but excluding those that died before week 52 (e.g. 4 animals had died within the first 12 months in the 25ppm group and are not evaluated for tumour burden); $ historical control data, 3 studies from 2005 – 2007; NB: significance of the trend is denoted by p values in the control or 0ppm column, significance of pairwise comparison with controls is denoted under each dose level.

Pair-wise comparison to the high dose male mice resulted in statistically significant increases for hepatocellular adenomas ($p < 0.05$), carcinomas ($p < 0.01$) and combined adenomas and/or carcinomas ($p < 0.01$). The incidences of adenomas, carcinomas and the combined tumours at the high dose exceeded the testing laboratories historical control mean range. High dose female mice had statistically significant trends for hepatocellular carcinomas ($p < 0.01$) and combined adenomas and/or carcinomas ($p < 0.05$). No statistical significance was seen in pair-wise comparisons for any tumour type. Although the incidence of carcinomas (9%) at the female high dose (1250 ppm) did not reach statistical significance, the incidences exceeded the male historical control mean (0.6%) and range (0-2%) for this malignant lesion.

Table 4.10.1.1.Study 2.8 (DAR Table 6.5.2.1-8b): Sulfoxaflor – CD-1 Mice: Female Liver Tumour Rates

Liver Lesion	0 ppm (0 mg/kg/day)	25 ppm (3.43 mg/kg/day)	250 ppm (33.9 mg/kg/day)	1250 ppm (176 mg/kg/day)
*total animals**	*46*	*46*	*48*	*45*
Adenomas	1/46	1/46	0/48	2/45
(%)	(2)	(2)	(0)	(4)
p =	0.227	0.753	0.000	0.492
Carcinomas	0/46	1/46	0/48	**4/45**
(%)	(0)	(2)	(0)	**(9)**
p =	0.009	0.500	0.000	**0.056**
Combined	1/46	2/46	0/48	5/45
(%)	(2)	(4)	(0)	(11)
p =	0.019	0.500	0.000	0.097
animals with both an adenoma and a carcinoma:	1	0	2	11
$adenomas (%)	2			
range (%)	0 – 6			
$carcinomas (%)	0.0			
range (%)	--			
$combined (%)	2			
range (%)	0 – 6			

*Tumour rates are based on number of tumour bearing animals relative to number of animals examined but excluding those that died before week 52 (e.g. 4 animals had died within the first 12 months in the 25ppm group and are not evaluated for tumour burden); $ historical control data, 3 studies from 2005 – 2007; NB: significance of the trend is denoted by p values in the control or 0ppm column, significance of pairwise comparison with controls is denoted under each dose level.

Conclusions

The mortality rate did not differ significantly between control and treated groups. There were no clinical signs observed after Sulfoxaflor exposure. The body weights of males and females exposed to all doses of Sulfoxaflor were unaffected by treatment. Similarly, there were no treatment-related changes in body weight gains, feed consumption, ophthalmological observations, and total and differential white blood cell counts.

Both male and female mice displayed a linear increased proportionality in sulfoxalor plasma concentration (and thus systemic dose) with increasing test material intake. Urinary elimination of Sulfoxaflor is also dose proportional for both sexes.

A number of statistically significant differences in absolute and relative organ weights were recorded at the end of the oncogenicity study. Most of these differences are not considered to be toxicologically significant; except in the case for high dose males and females where liver weights showed clear treatment responses. The mean absolute and relative adrenal gland weights of high dose females were slightly higher and statistically significant when compared to their respective controls (but still within the performing laboratory's recent historical control range). This finding was considered unrelated to treatment due to the absence of any associated clinical signs and histopathological findings. Unlike the situation in the rat oncogenicity study, testes weights were unremarkable.

The liver was the primary target of Sulfoxaflor. The absolute and relative liver weights of

high dose males were increased 87 and 79% respectively; in high dose females they were increased 51 and 47%, respectively when compared to controls. At necropsy, there was a treatment-related increase in the incidence of mass nodules and multifocal pale foci in the liver of males given 750 ppm. Females from the 1250 ppm group had a treatment-related increased incidence of mass nodules in the liver, albeit at lower numbers compared to the high-dose males. Histopathologic treatment-related changes consisted of hepatocellular adenomas and/or carcinomas in 60% of high dose male mice and in 11% of high dose female mice. Although there were no statistically significant differences in the overall mortality rates between the controls and any of the Sulfoxaflor treated groups, hepatocellular carcinomas or adenomas were attributed as the cause of death or moribundity in a small proportion of high dose males (6 of 50). Treatment-related non-neoplastic liver effects consisted of increases in the incidences of eosinophilic and vacuolated foci of cellular alteration in males given 750 ppm; slight to moderate centrilobular/midzonal or panlobular hepatocellular hypertrophy with altered tinctorial properties (increased cytoplasmic eosinophilia) consistent with liver enzyme induction in males and females given 750 or 1250 ppm; very slight or slight multifocal individual cell necrosis of hepatocytes in males given 750 ppm and females given 1250 ppm (very slight); very slight fatty change in centrilobular/midzonal hepatocytes in males and females given 750 or 1250 ppm and increased incidence of hepatocytes in mitosis in the male high dose group.

The only treatment-related change in mid dose females (250ppm) was an increased incidence of slight centrilobular/midzonal hepatocyte hypertrophy with altered tinctorial properties (increased cytoplasmic eosinophilia) consistent with liver enzyme induction. There were no other associated changes such as an increase in liver weight or other treatment-related histopathological findings.

Dermatitis affecting one or more sites was observed in some mice across all treated groups including controls. The higher incidence of dermatitis in high dose males (and to a much lesser extent in high dose females), may be interpreted as an exacerbation of an already spontaneous background lesion. Histologically, these were characterized by subacute to chronic inflammation, variable epidermal ulceration, acanthosis and an increased incidence of reactive plasmacytosis of the local submandibular lymph nodes. It is difficult to say what contribution if any, is made by exposure to Sulfoxaflor. In females, a non-statistically significant increase in lymphosarcoma is not thought to be toxicologically relevant.

Further studies (dual CAR/PXR knockout mice, gene expression with real-time PCR and Ki-67 immunohistochemical staining, liver enzyme analysis and molecular markers of PB-like activity and nuclear receptor analysis, described later) and a formal framework analysis investigating the proposed mode of action (MoA) support a threshold-based, mitogenic response similar to a phenobarbital (PB) like MoA for these liver tumours.

Based on treatment-related effects on the liver (increased liver weights, increased incidence of liver nodules, liver hypertrophy, and liver histopathology with eosinophilic and vacuolated foci) including a high incidence of hepatic tumours at 79.6 mg/kg bw/day (750ppm), the no observed adverse effect level (NOAEL) for males is 10.4 mg/kg bw/day (100ppm). The NOAEL for females is 33.9 mg/kg bw/day (250ppm), based also on liver effects at the highest tested dose at 176 mg/kg bw/day (1250ppm).

4.10.1.2 Carcinogenicity: inhalation

No data available.

4.10.1.3 Carcinogenicity: dermal

No data available.

4.10.2 Human information

No data available.

4.10.3 Other relevant information

4.10.3.1. Mechanism of action studies to address liver tumours in rodents

A treatment-related, statistically significant increase in the incidence of hepatocellular adenomas was seen in male rats in the high dose group of the rat 2-year study. High dose female rats did not have a treatment-related increase in the incidence of liver tumours. There were no treatment-related liver effects in lower dose males or females. Further studies (dual CAR/PXR knockout mice, gene expression with real-time PCR and Ki-67 immunohistochemical staining, liver enzyme analysis and molecular markers of PB-like activity and nuclear receptor analysis, described later) and a formal framework analysis investigating the proposed mode of action (MoA) support a threshold based, mitogenic response similar to a phenobarbital (PB) like MoA for these liver tumours. These tumours are considered to be rodent specific with little relevance to human hazard assessment.

At necropsy in the mouse 18 month study, there was a treatment-related increase in the incidence of mass nodules and multifocal pale foci in the liver of high dose males. High dose females also had a treatment-related increased incidence of mass nodules in the liver. Histopathological treatment-related changes consisted of hepatocellular adenomas and/or carcinomas in 60% of high dose male mice and in 11% of high dose female mice. Further studies (dual CAR/PXR knockout mice, gene expression with real-time PCR and Ki-67 immunohistochemical staining, liver enzyme analysis and molecular markers of PB-like activity and nuclear receptor analysis, described here) and a formal framework analysis investigating the proposed mode of action (MoA) support a threshold-based, mitogenic response similar to a phenobarbital (PB) like MoA for these liver tumours.

Study 1: Ex vivo gene expression and cell proliferation analyses in rats and mice. DAR Section B.6.5.3.1.

Report: Geter, D.R. and Kan, H.L. (2008). Gene Expression and Cell Proliferation Analyses in X11422208 Exposed Rats and Mice. Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan, 48674

Report No.: Study ID: 070158. US EPA MRID 47832033
Dates: Feb 2008
Guidelines: Non-guideline.
GLP: No. All experiments were done according to GLP standards and are fully reliable even though the study is not GLP compliant.
Deviations: This is acceptable as a basic though non-guideline study, it is considered supplementary to the long-term chronic / carcinogenicity studies. It reports on the analysis of samples taken from previous dietary studies.
Deficiencies: Yes, only livers from female CD1 mice were analysed with respect to specific gene expression profiling and cell proliferation. The male mouse is much

more sensitive and more likely to show an effect at a specific dose level. Gene expression profiling in the female mice was conducted on liver samples from animals exposed for 3 days and not the original study period of 7 days due to palatability issues.

Executive Summary: The purpose of this study was to obtain preliminary information on the potential mode of action responsible for the liver effects observed in mice and rats from the long term studies where animals were administered dietary Sulfoxaflor. Briefly, in carcinogenicity studies in mice and rats, an increased incidence of hepatocellular tumours were identified in male rats and male and female mice. The postulated mode-of-action (MoA) for these Sulfoxaflor induced liver tumours is via a nuclear receptor-mediated mode-of-action (MoA) through the following key events: (1) constitutive androstane receptor (CAR) receptor activation and (2) increased hepatocellular proliferation, leading to (3) hepatocellular tumours. Activation of rodent CAR and minor contributions of the pregnane X receptor (PXR) produces a cascade of alterations in gene transcription that leads to increased hepatocellular proliferation, a critical event in the development of liver tumors, similar to the established MoA for phenobarbital (PB).

This report describes (1) specific gene expression as assessed by real-time PCR in liver samples from female CD1 mice exposed to 0 and 4500ppm (345 mg/kg bw/day) dietary Sulfoxaflor for 3 days (section B.6.3.1/3a; study id 060523; *Thomas & Dryzga, 2007*); (2) cell proliferation assessed by Ki-67 immunohistochemical staining in liver tissue from 0 and 2000ppm group male and female F344 rats (155 and 170 mg/kg bw/day respectively) from the oral 28-day rat study (section B.6.3.1/2; study id 061170; *Yano et., al., 2007*) and (3) cell proliferation assessed by Ki-67 immunohistochemical staining in CD1 mouse liver tissue from 0, 3000, and 4500ppm dose groups (0, 418 and 345 mg/kg bw/day, final dose is lower due to decreased feed consumption) from the mouse palatability study (section B.6.3.1/3a; study id 060523, *Thomas & Dryzga, 2007*).

Background: A phenobarbital (PB) like mode of action (MoA) has been postulated for Sulfoxaflor induced rodent liver effects including increases in liver weight and tumour incidence. Typically, PB-induced liver enlargement and tumours involve the activation of the constitutive androstane receptor (CAR), induction of cytochrome P450 Cyp2b enzymes, particularly *Cyp2b10* in mice, hepatocellular hypertrophy, increased hepatocellular proliferation and the development of altered hepatic foci.

Results: Preliminary results indicate Sulfoxaflor induces a phenobarbital (PB)-like gene expression response consistent with CAR and PXR mediated induction of marker genes such as *Cyp2b10* (increased > 148 fold) and *Cyp3a11, Alas1, and NADPH-Cyp-reductase*. Sulfoxaflor stimulated the cholesterol synthesis-related genes, *Dhcr7* and *Sqle1*, and is not acting as a peroxisome proliferator. Sulfoxaflor increased liver hepatocyte proliferation in mice but weakly in rats: seen in the centrilobular region alone for rats and both the centrilobular and midzonal regions in mice.

Study 2: Targeted gene expression, cell proliferation and cytochrome P450 enzymatic activity in rats. DAR Section B.6.5.3.2.

Report: Geter, D.R., and Card, T.L. (2010). XR-208: Targeted gene expression, cell proliferation and cytochrome P450 enzymatic activity in rats. Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan, 48674.
Report No.: Study ID: 070339. DECO HET DR-0404-3134-029.
Dates: June 2010

Guidelines: Non-guideline.
GLP: No. All experiments were done according to GLP standards and are fully reliable even though the study is not GLP compliant.
Deviations: None. This is acceptable as a basic though non-guideline short term MoA study, it is considered supplementary to the long-term chronic / carcinogenicity studies.
Deficiencies: No.
Executive Summary: In previous studies targeted gene expression data in mice and hepatocellular proliferation data in both mice and rats indicated a possible phenobarbital (PB)-like mode of action (MoA) could be responsible for the liver effects related to Sulfoxaflor treatment. The purpose of this study was to determine if a PB-like MoA was responsible for the liver weight increases seen in Fischer 344 rats and to obtain information if any on dose responses of the effect. An additional aim of this study was to determine if other nuclear receptors in addition to CAR/PXR might have played a role in Sulfoxaflor-induced liver effects, namely; the aryl hydrocarbon receptor (AhR) and the peroxisome proliferator-activated receptor alpha (PPARα). Briefly, 5 male and 5 female Fischer 344/DuCrl rats per dose group were fed Sulfoxaflor in the diet at 0, 100, 750, and 1500ppm for 3 (0, 8.85, 60.3, and 99.2 mg/kg/day for males; 0, 7.83, 50.6, and 83.3 mg/kg/day for females) or 7 days (0, 8.02, 58.6, and 102 mg/kg/day for males; 0, 7.74, 53.1, and 94.4 mg/kg/day for females). The primary endpoints examined in this study were liver weight, targeted gene expression, liver enzyme analysis, and hepatocellular proliferation.
There was decreased body weight and body weight gains in males and females at the highest dose of 1500ppm after 3 and 7 days. Decreased food consumption in males and females at 750 and 1500 ppm after 3 days and in the 1500ppm group only after 7 days. There was elevated cholesterol levels in males at 750 and 1500ppm after 3 and 7 days of treatment but elevated cholesterol levels in females were only observed at 1500ppm after 7 days. At 1500ppm after 3 days the relative liver weights were increased for males only (14%), females showed a slight effect (3%); at 750 and 1500ppm after 7 days the relative liver weights were increased by 11 and 23% for males with lower increments of 6 and 18% for females, respectively. *Cyp2b1* gene expression, the prototypical gene response following PB exposure, was induced over 800-fold in both male and female rats exposed to 1500 ppm Sulfoxaflor for 3 and 7 days. *Cyp2b2* and *Cyp3a3* (CAR- and PXR-related genes, respectively) expression levels, together with PROD and BROD enzyme activity were increased for all animals in the 750 and 1500ppm dose groups on both test days in support of a PB-like response in rodent liver. Significant hepatocellular proliferation was observed in males and females on the 2 highest doses on day 7.

Cyp1a1 gene expression and EROD enzyme activity were slightly but significantly elevated at day 3; however, EROD enzyme activity returned to control levels by day 7. In addition, gene expression of *Cyp4a22* was not elevated in this study. These results indicate no agonism or activation of the AhR or PPARα nuclear receptors. Overall, the results support the activation of CAR with contributions of the pregnane X receptor (PXR) in rodent liver when animals are exposed to Sulfoxaflor.

Based upon these results, increased liver weight in rats administered dietary Sulfoxaflor was similar to the action of phenobarbital, as evidenced by the CAR and PXR-related molecular, enzymatic, and proliferative responses.

Study 3: Mode of Action Study Investigating Liver Weight Effects in Crl:CD-1(ICR) Mice. DAR Section B.6.5.3.3.

Report: Geter, D. R., Murray, J. A., L.V.T., Kan, H. L., LeBaron, M. J. and Thomas, J.

(2010). XDE-208: Mode of Action Study Investigating Liver Weight Effects in Crl:CD-1(ICR) Mice. Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan, 48674.

Report No.: Study ID: 080246. DECO HET DR-0404-3134-041.

Dates: March 2008

Guidelines: Non-guideline.

GLP: No. All experiments were done according to GLP standards.

Deviations: None. This is acceptable as a basic though non-guideline short term MoA study, it is considered supplementary to the long-term chronic / carcinogenicity studies.

Deficiencies: No.

Executive Summary: In previous studies limited targeted gene expression data in mice, a more comprehensive study of targeted gene expression in rats and hepatocellular proliferation data in both mice and rats indicated a possible phenobarbital (PB)-like mode of action (MoA) could be responsible for the liver effects related to sulfoxaflor treatment. The purpose of this study was to investigate in further detail if a PB-like MoA was responsible for liver weight increases seen in CD-1 mice following sulfoxaflor exposure and to obtain information on a possible dose response of the effect or if the effects follow on from a threshold limit. In concert with the rat study described previously (section B6.5.3.2); an additional aim of this study was to determine if other nuclear receptors in addition to CAR/PXR might have played a role in sulfoxaflor-induced liver effects, namely; the aryl hydrocarbon receptor (AhR) and the peroxisome proliferator-activated receptor alpha (PPARα).

Briefly, 5 male and 5 female CD-1 mice per dose group were fed sulfoxaflor in the diet at either 0, 500, and 750ppm for males (0, 89, and 128mg/kg bw/day), or 0, 1000, and 1500ppm for females (0, 211, and 323mg/kg bw/day) for a total of 7 days. The primary endpoints examined in this study were liver weight, targeted gene expression, liver enzyme analysis, and hepatocellular proliferation. In addition, archived liver samples from previously conducted 28 and 90-day sulfoxaflor mouse studies were analysed for targeted gene expression, liver enzyme activity, and hepatocellular proliferation (Ki-67).

Liver weights increased with treatment dose of sulfoxaflor. High dose males (750ppm) had an absolute liver weight increase of 14% (17% in relative liver weight) compared with controls. The effect was greater in females on higher dose treatments, mean group liver weights increased by 43% and 47% (38% and 43% for relative liver weight) in animals exposed to dietary levels of 1000ppm and 1500ppm respectively. These liver weight increases correlated with treatment-related observations of centrilobular and midzonal hepatocyte hypertrophy with very slightly increased cytoplasmic eosinophilia. There was also evidence of lipid changes in the hepatocytes of high dose males, and increased numbers of mitotic hepatocytes and individual cell necrosis at doses ≥ 500ppm in both sexes.

An elevation in *Cyp2b10* levels was characteristic of all animals exposed to sulfoxaflor and liver samples from the 28 and 90-day studies. Males generally had a higher response than females, i.e. they were more sensitive, even though their systemic exposures were lower. These results for *Cyp2b10* mRNA concurred with increased PROD and BROD liver enzyme activities in all animals on all doses. Similarly, *Cyp3a11* levels were also elevated in high dose males and all female dose groups. Hepatocyte proliferation was also evident from

results using the BrdU and Ki-67 immunohistochemical techniques. Ki-67 analysis of hepatocellular proliferation was less sensitive then BrdU, in contrast to the BrdU results, increased proliferation was not evident at any dose or zone by Ki-67 analysis. Ki-67 analysis of hepatocellular proliferation in the 28 and 90-day studies showed no induction at either time point in male or female mice.

AhR-related EROD liver enzyme activity was slightly elevated in this study at all time-points in both male and female mice; however, the degree of induction was mild (none greater than 2.3-fold) and may be associated with the large induction of Cyp2b enzyme. *Cyp4a10*, a PPARα related gene, was not significantly altered in this study.

Overall, the results support the idea of a PB-like response by the liver when animals are exposed to sulfoxaflor.

Based upon these results, increased liver weight in mice administered dietary sulfoxaflor appears to be similar to the action of phenobarbital, as evidenced by the CAR and PXR-related molecular, enzymatic, and proliferative responses.

Study 4: Mechanism of action Study: Mouse strain suitability. (DAR Section B.6.5.3.4.)

Report:B. M. Elcombe. (2010). XDE-208: A Study to Characterize the Induction Profile of XDE-208 in the Livers Of C57BL/6J Mice. CXR Biosciences Ltd., James Lindsay Place, Dundee Technopole, Dundee, DD1 5JJ and Medical School Resource Unit (MSRU), Dundee University, Dundee, DD1 9SY.

Report No.: Study ID: CXR0821. DECO HET DR-0404-3134-116

Dates: May 2009

Guidelines: Non-guideline.

GLP: No. However, all experiments were done according to GLP standards.

Deviations: None. This is acceptable as a basic though non-guideline short term MoA study, it is considered supplementary to the long-term chronic / carcinogenicity studies and critical to ensuring that data from an extensive study using C57BL/6J CAR/PXR knockout and humanised mice is comparable with the data generated in previous studies which utilised CD1 mice exposed to sulfoxaflor.

Deficiencies: Yes in that it would have been a more complete study to characterise the hepatomegaly with liver histopathology data because this effect is typically characterised by hepatocellular hypertrophy and hyperplasia in the short term.

Executive Summary: In previous studies, limited targeted gene expression data has been generated in the Crl:CD1(ICR) mouse strain to support the hypothesis that sulfoxaflor acts through a phenobarbital (PB)-like mode of action (MoA) involving activation of the CAR receptor. The present study seeks to validate the suitability of an alternate mouse strain (C57BL/6J) with respect to liver enzyme induction, gene expression and proliferative responses to dietary sulfoxaflor. A more comprehensive study of the role of the CAR/PXR receptors can be studied with the use of CAR/PXR knockout and humanised mice but these experimental models are only available in the C57BL/6J strain.

Sulfoxaflor was administered in the diet to 5 male C57BL/6J mice per dose group at dose levels of 0, 750, or 1500 ppm (equivalent to 0, 160, and 310 mg/kg/day respectively) for 7

days. The primary endpoints examined in this study included daily clinical observations, body weights, body weight gain, feed consumption, serum clinical chemistries, focused gene expression and protein quantification, liver cytochrome P450 enzyme activity, and hepatocellular proliferation. Males only were selected as they are more sensitive to the effects of sulfoxaflor. The dose levels selected were based upon previous studies in CD1 mice.

Sulfoxaflor administration resulted in hepatomegaly. There was a treatment-related increase in absolute and relative liver weights following seven days of exposure to sulfoxaflor. There was no evidence of hepatotoxicity at any dose level. Treated animals did have raised plasma ALT levels, there was a dose-dependent and statistically significant increase in ALT (< 2-fold in the high dose group relative to controls) but it is not considered toxicologically significant. There were also minor increases in both AST and triglycerides for the high dose group alone with little to no change in cholesterol levels amonst all dose groups. Cytochrome P450 activity increased with sulfoxaflor dose. Administration of sulfoxaflor at 750ppm and 1500ppm elicited a 3- to 5- fold increase in total hepatic P450, respectively, a 33-fold increase in PROD activity at both concentrations, a 47- and 82- folding increase in BROD activity, respectively, and a 4-and 7-fold increase in BQ activity, respectively

Elevations in *Cyp2b10* and *Cyp3a11* gene expression levels were observed. The strongest response was associated with the expression of *Cyp2b10* mRNA, going from indeterminant (i.e. very low) levels in controls to a positive dose response increment of 9-fold between the 2 doses tested. Unlike *Cyp2b10*, *Cyp3a11* is constitutively expressed in this strain of mouse (C57B1/6J), and results can be expressed as a relative fold change over control values. Administration of 750 and 1500 ppm XDE-208 resulted in a 2.4-and 5.6-fold increase in *Cyp3a11* relative to controls. Sulfoxaflor induced gene expression data was confirmed by investigation of the resultant gene products, i.e. proteins via sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting.

Overall, the data supports inter-strain comparisons to data previously obtained from studies in CD1 mice and suggests an involvement of the CAR/PXR nuclear receptor system in the consequent liver effects seen with sulfoxaflor exposure.

Based upon these results, increased liver weight in C57BL/6J mice administered dietary sulfoxaflor appears to be similar to the action of phenobarbital, as evidenced by the CAR and PXR-related molecular and enzymatic responses and is comparable to those seen in other rodent species and genetic strains.

Study 5: MoA Study: Mouse/C57Bl/6J WT, Humanised and KO PXR/CAR transgenic models. (DAR Section B.6.5.3.5.)

Report: Ross, J. XDE-208 (2010): A Study To Investigate The Mode of Action For Liver Effects Observed In Regulatory Toxicology Studies By Use of Dual Car-PXR Knockout And Humanised Mice.CXR Biosciences Ltd., James Lindsay Place, Dundee Technopole, Dundee, DD1 5JJ and Medical School Resource Unit (MSRU), Dundee University, Dundee, DD1 9SY.

Report No.: Study ID: CXR0867. DECO HET DR-0404-3134-112.

Dates: 2009

Guidelines: Non-guideline.

GLP: No. However, all experiments were done according to GLP standards.

Deviations: None. This is an acceptable though non-guideline short term MoA study, it is considered supplementary to the long-term chronic / carcinogenicity studies and critical to illustrating the roles of the CAR/PXR nuclear receptors in mediating the effects of sulfoxaflor on the rodent liver as well as accounting for species differences in liver response.

Deficiencies: None. General comments: no definitive distinction between CAR and PXR activities because double knockout mice and double humanised PXR-CAR mice were used in this study.

Executive Summary: In previous rodent studies hepatomegaly characterised by hepatocellular hypertrophy and hyperplasia in the short term, and, at high doses, hepatocellular tumours in the long term, is a feature of sulfoxaflor exposure. Limited targeted gene expression data indicates similarities to gene expression events promoted by phenobarbital which is known to activate the CAR receptor. So called "humanised" and knockout PXR and CAR mouse models have been utilised to investigate the effects of xenobiotics on the liver as it is wellknown that CAR/PXR are involved in the apparent species differences in the stimulation of the hyperplastic response. The CAR/PXR knockout models can be used to identify whether the mechanism of action is CAR/PXR-dependent and therefore potentially similar to the effects caused by phenobarbital. Phenobarbital is an example of a mouse non-genotoxic carcinogen that according to all reports so far, does not cause cancer in humans. The use of "humanised" CAR/PXR animals obliterates the proliferative or hyperplastic response normally seen in wild type animals exposed to phenobarbital and raises questions whether xenobiotics such as phenobarbital pose a hepatocarcinogenic hazard to humans.

The purpose of the study was to investigate: (1) if the constitutive androstane receptor (CAR) and pregnane X receptor (PXR) mediate sulfoxaflor-induced hypertrophy and hyperplasia in mice; and (2) if the human orthologs support these processes to a similar extent as the murine receptors. The mouse models used were wild type C57BL/6J (WT) mice, C57BL/6J mice null for PXR and CAR (PXRKO/CARKO) and C57BL/6J mice "humanised" for PXR and CAR (hPXR/hCAR). Sulfoxaflor was administered in the diet to 10 male rats of each strain at a dose level of 750ppm (equivalent to 115.6, 120.4 and 99.3mg sulfoxaflor/kg body weight/day, for WT, PXRKO/CARKO and hPXR/hCAR mice respectively) and 0 dose controls for 7 days. Parameters examined included: daily clinical observations, body weights, body weight gain, feed consumption, plasma clinical chemistries, focused gene expression, protein quantification, liver cytochrome P450 enzyme activity, hepatocellular proliferation using nuclear incorporation of BrdU, and liver histopathology.

There were no treatment-related clinical observations or effects on body weight or body weight gain in any strain of mouse. There were treatment-related increases in absolute (24% and 9% respectively) and relative (25% and 12% respectively) liver weights in WT and hPXR/hCAR mice but not in the PXRKO/CARKO animals. In WT mice, sulfoxaflor treatment increased hepatocellular proliferation (approximately 4-fold) but no such changes in proliferation were seen in either the hPXR/hCAR or PXRKO/CARKO mice. Treatment-related hepatocyte hypertrophy was observed in WT and hPXR/hCAR mice while increased mitotic figures were observed only in WT mice (the knockouts failed to show either response).

Sulfoxaflor behaved as a phenobarbital-like inducer in WT mice (marked induction of total cytochrome P450, increased PROD and BROD, increased expression of *Cyp2b10* mRNA, and increases in Cyp2b10 protein. However, in the hPXR/hCAR under the same conditions, induction of Cyp2b10 activity, protein and mRNA was markedly less than observed in the WT animals following treatment with sulfoxaflor. Sulfoxaflor had no significant effect on *Cyp2b10* expression or catalytic activity in the genetic knock outs.

Similar sulfoxaflor-mediated *Cyp3a11* induction, as determined by BQ activity, RT-PCR and immunoblotting was observed in the "humanised" and WT mice, but was not seen in the PXRKO/CARKO mice.

The results suggest sulfoxaflor exhibits more activity towards the mouse CAR/PXR than the human CAR/PXR and that the CAR/PXR receptors are intimately tied into the liver response resulting from sulfoxaflor exposure. Additionally, the data show that the human CAR/PXR support sulfoxaflor-induced hypertrophy but not hyperplasia thus indicating species susceptibility differences due to the CAR/PXR genotype present.

In summary, Sulfoxaflor exhibited greater activity towards the mouse CAR / PXR than the human CAR / PXR. The difference in hepatic response between wild type and humanised mice in this study is considered to be mediated via species specific features of CAR / PXR. The data shows that the human CAR / PXR construct supports sulfoxaflor-induced hypertrophy but not hyperplasia, a situation similarly seen with phenobarbital exposure in humans. The hyperplastic response is thought to be a major contributing factor in determining the potential for hepatocellular carcinogenesis in rodents. This study demonstrates that a significant species response is due to the CAR / PXR genotype present and questions the relevancy of sulfoxaflor-induced liver tumours in rodents with respect to liver tumour risk in humans where it may not act as a liver carcinogen.

Study 6: Human Relevance Framework for Liver Tumours. DAR Section B.6.5.3.6.

Report: LeBaron, M.J., Rasoulpour, R.J., Geter, D.R., Billington, R. and Gollapudi, B.B. (2010). XDE-208: Mode of action and human relevance framework analysis for XDE-208-induced rodent liver tumors. Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan, 48674

Report No.: Study ID: 100291. DECO HET DR-0404-3134-118
Dates: 2010
Guidelines: Not applicable. Not required for EU dossier submission.
GLP: Not applicable.
Deviations: None. This is an acceptable overview of all the data presented thus far in section B6.5 as pertains to sulfoxaflor-induced liver tumours in rodents and the toxicological relevancy of this effect to man.
Deficiencies: None. General discussion document.
Executive Summary: Sulfoxaflor causes liver tumours in rodent carcinogenicity studies via a proposed nuclear receptor-mediated mode-of-action (MoA) through the following key events:

(1) CAR receptor activation and;

(2) Increased hepatocellular proliferation, leading to

(3) Hepatocellular tumours.

These key events have been evaluated in a series of MoA studies aimed at examining the causality of sulfoxaflor's induction of liver tumours in the chronic studies. This document represents the weight of evidence approach used to evaluate the data based upon the Bradford-Hill criteria followed by subsequent application in a Human Relevance Framework (HRF). The conclusion from this evaluation is that the observed sulfoxaflor-induced rodent liver tumours occur via a CAR-mediated MoA for which there is a high level of confidence. Activation of rodent CAR (and minor contribution of PXR) produces a cascade of alterations in gene transcription that leads to increased hepatocellular proliferation, a critical event in the development of liver tumours, and similar to the established MoA for phenobarbital (PB). On the other hand, PB in humans results in activation of CAR and PXR leading to the induction of cytochrome P450 (CYP) enzymes; however, different enzymes are induced in humans compared to rodents and, more importantly, there is no evidence of increased hepatocellular proliferation in humans. Furthermore, extensive epidemiologic studies in humans exposed to levels of PB comparable to those in rodent bioassays did not find an increased risk of liver cancer. This finding was reinforced in the course of these studies with sulfoxaflor, where humanised CAR/PXR knock-in mice were refractory to the hepatocellular proliferative effect of sulfoxaflor, whereas wild-type mice demonstrated increased proliferation (section B6.5.3.5). Based on a previous MoA assessment, PB is not a hepatocarcinogen in humans. Furthermore, a hepatocarcinogenic response in rodents for compounds which have data to support a PB-like MoA is not relevant to humans. On this basis, the rodent liver tumours associated with administration of high dose levels of sulfoxaflor would not pose a cancer hazard to humans.

Conclusions:

Statement of confidence in the evaluation.

This Human Relevance Framework evaluation for sulfoxaflor-induced hepatocellular tumours in mice and rats follows the guideline established for this process (*Sonich-Mullin et al., 2001; Cohen et al., 2003; Meek et al., 2003; USEPA, 2005; Holsapple et al., 2006; Boobis et al., 2007*). The extensive toxicological database for sulfoxaflor, including several focused MoA studies in both mice and rats, as well as a study in genetically-engineered (knockout and humanised) mice are high quality studies that provide the necessary data to determine the MoA for sulfoxaflor-induced rodent liver tumours.

Key event #1 for the sulfoxaflor-induced liver tumour MoA is defined as activation of the CAR nuclear receptor, which is measured by the induction of *Cyp2b*/CAR-associated transcript (*Cyp2b10* in mice and *Cyp2b1* in rats), protein, and liver enzymatic activity. The *Cyp2b*/CAR-associated transcript and protein data define a very specific MoA while, at the same time, the data rule out several other potential nuclear receptor-mediated MoAs for rodent hepatic carcinogens such as PPAR-α and AhR agonism. PXR nuclear receptor-mediated *Cyp3a* cytochrome induction (*Cyp3a11* in mice and *Cyp3a3* in rats) was slightly induced after sulfoxaflor administration, analogous to the response after treatment with PB and consistent with the well documented co-activation of the receptors. Furthermore, these results were shown to be dependent on the rodent CAR and PXR nuclear receptors as knockout and humanised mice were not similarly responsive to sulfoxaflor treatment. Supportive, associative key events to #1 include increased liver weight and microscopic hepatocellular hypertrophy.

Key event #2 is an increase in hepatocellular proliferation and was indentified in both mice and rats. Importantly, neither the CARKO/PXRKO or hCAR/hPXR mice had increased

hepatocellular proliferation, underscoring the difference of rodent and human responses to CAR activation, and the qualitative differences in nuclear receptor-mediated hepatic responses. The key events for sulfoxaflor show clear, threshold, dose-responsive alterations and provide informative, temporal-specific characterisation of sulfoxaflor-induced liver effects. These key events are consistent with a CAR-mediated (PB-like) MoA. The concordance analysis points out clear differences for a PB-like MoA in rodents as compared to humans. A hepatocarcinogenic response in rodents for compounds that have data to support a PB-like MoA, such as sulfoxaflor, is not relevant to humans (*Holsapple et al., 2006*).

Other possible MoAs for hepatocellular carcinogenesis as described by *Cohen* (2010) have been evaluated with respect to sulfoxaflor. Other MoAs due to increased cell proliferation (including receptor-mediated and non-receptor-mediated) or DNA reactivity have been dismissed for sulfoxaflor hepatocellular tumours because they lack plausibility and coherence or, in the case of cytotoxicity, because of the lack of coherence when the dose response for cytotoxicity is compared to the hepatocellular tumour dose response.

Identification of data gaps. Male mice and rats were more sensitive to the hepatic effects of sulfoxaflor and, hence, most of the mechanistic evaluations for MoA were performed in male mice and rats, including the studies with genetically engineered mouse models. Accordingly, the MoA/HRF evaluation described herein focused on the evaluation of the MoA in male mice and rats, although hepatocellular tumours at a lower incidence than that in their male counterparts were identified in female mice treated with a higher dietary concentration of 1250ppm for 18 months. Histopathological examination of the liver of those animals at dose levels with hepatocellular tumours (and of liver tissue in the shorter duration studies) revealed a phenotype entirely consistent with that identified in males of increased cytochrome P450 induction and eosinophilia. While inclusion of females in the MoA studies and MoA evaluation may have been informative, the MoA data provide compelling evidence that the sulfoxaflor liver tumour MoA is not sex specific but is sex selective in that males are more sensitive even at lower doses. Restricting the MoA investigations to the more sensitive sex significantly reduced the number of animals used for the studies.

Reversibility of sulfoxaflor-induced hepatic effects was investigated in a standard, repeat dose 90-day rat toxicity study. Animals administered the top dietary concentration of 1500ppm (i.e., 3-fold greater than the hepatocellular carcinogenic dose level in the 2-year rat study) for 90 days had a relative liver weight increase of 41% with clear microscopic hepatocellular hypertrophy identified. A subset of these animals were then subsequently switched to a control diet for an additional 28 days and the data indicated those animals did not have significantly increased relative liver weights or microscopic hepatocellular hypertrophy compared to control. A complete evaluation of the molecular reversibility for sulfoxaflor-induced hepatic effects across all MoA studies was not undertaken in an effort to restrict animal usage, as the most definitive experiment for specificity of sulfoxaflor-induced liver effects was demonstrated with the use of CARKO/PXRKO (knockout) and hCAR/hPXR (humanised) mice. The data from those animals demonstrated the molecular basis for the hepatocellular effects of sulfoxaflor.

Implications for risk assessment: There is convincing evidence that the MoA for sulfoxaflor-induced hepatocarcingenic effects in the mouse and rat liver do not occur below a defined dose level. Specifically, the MoA key events and hepatocellular tumours only occur at dietary concentrations greater than 100 ppm in the mouse and rat, and tumours were noted at 500 and 750 ppm, respectively. Furthermore, a hepatocarcinogenic response in rodents for

compounds that have data to support a PB-like MoA, such as sulfoxaflor, is not relevant to humans (*Holsapple et al., 2006*). These data were strengthened by the lack of hepatocellular proliferation in the CARKO/PXRKO and hCAR/hPXR mice. On this basis, the mouse and rat liver tumours associated with administration of higher dose levels of sulfoxaflor would not pose a cancer hazard to humans. Based on this hazard assessment for the sulfoxaflor-induced mouse and rat liver tumours, a margin of exposure risk assessment based on the reference dose (RfD) would be protective of human health.

4.10.3.2. Mechanism of action studies to address Leydig cell tumours in rodents.

Study 7: MoA Study: Rat/F344 and Crl:CD(SD); (testosterone elimination and dopamine agonism and / or enhancement MoA study. DAR Section B.6.5.4.1.

Report: Rasoulpour, R. J., Zablotny, C. L., Clark, A. J., Hansen, S. C., Zhang, F. (2010). XDE 208: Leydig Cell Mode-of-Action Study in Crl:CD(SD) and F344/DuCrl Rats. Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan, 48674. Unpublished.

Report No.: DECO HET DR-0404-3134-115. Study ID: 101105.

Dates: 2010

Guidelines: Non-guideline.

GLP: Yes. All experiments were done according to GLP standards and are fully reliable even though the study is not GLP compliant.

Deviations: This is an acceptable though non-guideline study, it is considered supplementary to the long-term chronic / carcinogenicity studies.

Deficiencies: Yes, a group of positive control animals treated with a known and well documented dopamine agonist (DA) such as mesulergine would have provided the appropriate positive data to relate results from sulfoxaflor treated animals and therefore give a better understanding into the actions of sulfoxaflor. This would help to determine if sulfoxaflor operated in a similar manner to a DA.

Executive Summary: In a recently conducted two-year rat carcinogenicity study, male Fischer 344 rats given 100 or 500 ppm sulfoxaflor had a treatment-related increase in testis weight due to increased Leydig cell tumour (LCT) size. Histopathological examination confirmed that there was no increase in the overall incidence of LCT across the groups with 88, 92, 90, and 92% of rats affected at 0, 25, 100, and 500 ppm, respectively. However, there was a significant increase in bilateral LCT incidence at 500 ppm. The objective of this study was to identify the mode-of-action (MoA) responsible for these Leydig cell effects, also to determine if the MoA operated in Crl:CD(SD) rats, the strain used in the two-generation reproductive toxicity study where an apparent slight delay in preputial separation was seen at the high dose level of 400ppm.

General modes of action for rat Leydig cell tumours: It is generally accepted in the literature that there are nine known modes-of-action for Leydig cell tumour induction in rats, which fall into three 'bins' of human relevance (i.e., relevant, low relevance, no relevance). These are:

Relevant to humans: (1) mutagenicity

Low relevance to humans:	(2) androgen receptor antagonism
	(3) oestrogen receptor agonism/antagonism
	(4) 5-alpha-reductase inhibition
	(5) aromatase inhibition
	(6) **reduced testosterone biosynthesis**
	(7) **increased testosterone biliary elimination**
No relevance to humans:	(8) GnRH (LHRH) agonism
	(9) **Dopamine agonism/enhancement**

Relevant modes of action for sulfoxaflor-induced LCTs: The only relevant modes of action for sulfoxaflor considered to operate are those points emboldened above (MoA #6, #7, and #9). The suite of toxicity studies on sulfoxaflor, from a battery of genetic toxicity assays to developmental and reproductive toxicity to chronic/carcinogenicity studies, provides evidence that either refutes or cast significant doubt on the plausibility of a number of the other MoAs. For example, MoA #1 (mutagenicity) is not plausible as sulfoxaflor was negative in all *in vitro* and *in vivo* genetic toxicity assays. In addition, MoA #2 – 5 and #8 are also not plausible as there were no effects on end points that would have been affected with these MoA, such as male anogenital distance, accessory sex gland weights, mating or fertility indices, vaginal patency, or pituitary effects.

Reduced testosterone biosynthesis as a primary effect (#6) was deemed to have low plausibility as there was an increase in serum cholesterol levels with sulfoxaflor administration and a slight delay in preputial separation; however, there was no effect on female reproductive parameters, which would have been expected with this MoA as androgens are the precursors to oestrogens. In support of MoA #9, a prototypical dopamine agonist/enhancer, such as mesulergine, would cause a delay in preputial separation as well as decreased levels of circulating Prl (*Prentice et al., 1992*). Despite the relatively low plausibility, an assessment of steroidogenic gene expression was performed in this study to evaluate the reduced testosterone biosynthesis MoA.

The two most plausible MoAs, which both had a detailed analysis in this LCT MoA study, were increased biliary elimination of testosterone (#7) and dopamine agonism/enhancement (#9). MoA #7 was deemed plausible due to known nuclear receptor-mediated liver effects of sulfoxaflor, which could result in increased biliary elimination of testosterone and a compensatory increase in luteinizing hormone (LH) release from the pituitary gland. Trophic stimulation of the rat Leydig cells by persistently higher levels of circulating LH would, over time, lead to formation of Leydig cell tumours (*Cook et al., 1999*). MoA #9 was deemed plausible because sulfoxaflor is an agonist to the foetal rat muscle nicotinic acetylcholine receptor (nAChR) (*Millar, 2010*), the molecular target for insecticidal activity is the nAChR, and mammalian central nAChRs are known to play a key regulatory role in dopamine release in the brain (*Maskos, 2010*). The dopamine agonism/enhancement MoA occurs via antagonist action of dopamine on prolactin (Prl) release in the pituitary gland (*Cook et al., 1999*). Lower circulating Prl results in decreased prolactin binding on rat Leydig cells, which results in down-regulation of the LH receptors (*Prentice and Miekle, 1995*). This, in turn, results in transient decrease in circulating testosterone, which feeds back to stimulate an increase in LH release from the pituitary. As with MoA #7, chronic LH stimulation can lead to Leydig cell

hyperplasia and eventually tumour formation.

Groups of 15 Fischer 344 and 15 Crl:CD(SD) rats were given 0, 25, 100, or 500ppm sulfoxaflor in diet (120 total animals) for up to 8 weeks. After two weeks of treatment, three rats / group were selected for the biliary elimination of testosterone (#7) portion of the study. Briefly, bile duct cannulated rats were injected with ^{14}C-testosterone followed by bile and plasma collection over a two-hour period to determine if sulfoxaflor treatment altered the biliary elimination profile. In order to directly test if dopamine agonism / enhancement (#9) was the responsible MoA, a serum hormone panel of testosterone (T), luteinizing hormone (LH) and prolactin (Prl) were evaluated on all available animals after 2, 4, and 8 weeks of treatment. In addition to hormone measurements, gene expression analysis for LH receptor (LHR) and Prl receptor (PrlR) was performed on testes of 4- and 8-week treated Fischer rats. To directly test if reduced testosterone biosynthesis (#6) was the responsible MoA, gene expression of critical steroidogenic enzymes StAR (steroidogenic acute regulatory protein), Cyp11a1 (P450side chain cleavage), Cyp17a1 (17alpha-hydroxylase), HSD3b (3-β hydroxysteroid dehydrogenase), and SDR5a1 (5-α reductase) were evaluated in 4- and 8-week Fischer rat testes. If reduced testosterone biosynthesis was the operant MoA, one or more of these genes would be affected.

Results from the biliary elimination portion of this study revealed no treatment-related differences in the mean ^{14}C-testosterone derived radioactivity excreted in the bile, levels in circulating plasma, or in bile flow for Crl:CD(SD) and Fischer rats. This refutes (#7) as the operant MoA. Reduced testosterone biosynthesis (#6) had low plausibility due to the fact that female reproductive parameters were not affected in any study, including the two-generation reproductive toxicity study. There were no effects such as altered oestrous cyclicity, mating and fertility indices. There were no dose-dependent effects of treatment on any measured gene in the steroidogenic pathway including *StAR*, *Cyp11a1*, *Cyp17a1*, *HSD3b*, or *SDR5a1*. If reduced testosterone biosynthesis was the operant MoA, one or more of these genes would have been affected. The data presented in this study provide evidence supporting (#9) in the form of decreased circulating Prl levels, with increased LH and T levels, along with decreased testis LHR gene expression. The observation of hormone level alterations in this study support a hormonally-mediated, and thereby threshold, nonlinear mode-of-action. This MoA is hypothesised to operate through sulfoxaflor-mediated enhancement of dopamine release, potentially though agonism of α4β2 or α4α6β2 central nicotinic acetylcholine receptors (nAChRs), which are known to play a key regulatory role in dopamine release from dopaminergic neurons in the brain.

It is hypothesised that the LCT promotion seen in the rat chronic/carcinogenicity study was through weak, but chronic, enhancement of dopamine release, and subsequent inhibition of prolactin release from the pituitary gland, ultimately leading to a dopamine agonism/enhancement LCT MoA in a uniquely susceptible animal model, the Fischer 344 rat. This MoA would be considered to have no relevance to humans, per se.

Based on the data presented in this study, it is plausible though not conclusive that the LCT promotion seen in the rat chronic/carcinogenicity study was through weak, but chronic, enhancement of dopamine release, and the subsequent inhibition of prolactin release from the pituitary gland, ultimately leading to a dopamine agonism/enhancement LCT MoA in a uniquely susceptible animal model, the Fischer 344 rat. This MoA would be considered to have no relevance to humans, per se. In addition to providing data to support or refute specific LCT MoA, the observation of hormone level alterations in this study are equivocal with respect to supporting a hormonally-mediated, and thereby threshold, nonlinear mode-of-action.

Study 8: Proof of Concept Study: Dopamine microdialysis experiment. DAR Section B.6.5.4.2.

Report: Rowley H. L. And Heal, D. J. (2011). Effects of sulfoxaflor infusion on hypothalamic dopamine, DOPAC and HVA efflux – a microdialysis experiment in freely moving rats. RenaSci Consultancy Ltd, BioCity Nottingham, Pennyfoot Street, Nottingham, NG1 1GF, UK. Unpublished.

Report No.: DR-0404-3134-124; Study ID: RS867.

Dates: 2011

Guidelines: Non-guideline.

GLP: No. Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were not provided.

Deviations: This is an acceptable though non-guideline study, it is considered supplementary to the long-term chronic / carcinogenicity studies and suitable for a MoA investigation.

Deficiencies: Yes. An extended variation of this study could have also easily investigated dopamine agonists and/or reuptake inhibitors as supplemental positive controls in addition to K^+ spiking that may more closely mimic the proposed *in vivo* effects of sulfoxaflor. In addition, more time should have been allowed in between infusion events to allow dopamine responses to return to near baseline levels.

Executive Summary: Sulfoxaflor is a nicotinic acetylcholine receptor (nAChR) partial/weak agonist in the rat that is postulated to increase dopaminergic neurotransmission in the tuberoinfundibular (TIDA) system. In turn, the increased release of dopamine (DA) into the hypothalamic portal circulation further inhibits the release of prolactin by the pituitary. This hypothesis was tested by measuring the effects of reverse dialysis of sulfoxaflor on the extracellular concentration of DA in the mediobasal hypothalamus of male SD rats (n = 7). Since the concentration of analytes crossing the semi permeable membrane of the microdialysis probe is approximately 10 fold lower than the concentration present in the perfusion fluid (assumed, not measured), sulfoxaflor was reverse dialysed at a concentration of 400µM in the external, artificial cerebrospinal fluid (to replicate a concentration of 40µM in the extracellular fluid of the mediobasal hypothalamus) and at the higher concentration of 2mM (to replicate a concentration of approximately 200µM in the extracellular fluid of the mediobasal hypothalamus). In addition to measuring the effect of sulfoxaflor on DA release, the extracellular concentrations of its two major metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were also determined. A depolarising pulse of

50mM K^+ ions was used as a positive control and enhancer of increased local dopaminergic activity. This pulse of K^+ ions produced a transient and sharply delineated increase in DA efflux confirming that these hypothalamic dopaminergic neurones were viable and normally responsive.

Sulfoxaflor (at external concentrations of 400µM and 2mM) produced dose related increases in the extracellular level of dopamine in the mediobasal hypothalamus. Relative to the initial baseline, the increases evoked by sulfoxaflor were 15.4% at 400µM and 25.8% at 2mM. Sulfoxaflor and K^+ ions increased the extracellular concentration of dopamine and produced concomitant reductions in the concentration of HVA; neither sulfoxaflor nor K^+ ions altered the extracellular concentration of DOPAC. The identical profiles of K^+ and sulfoxaflor indicate that sulfoxaflor was causing an increase in local, external dopamine concentrations from the hypothalamic dopaminergic neurones. Since a concentration of sulfoxaflor of 400µM in the dialysis perfusion fluid equates to an extracellular concentration of approximately 40µM, it is reasonable to hypothesise that *in vivo* a circulating concentration of $\geq$ 40µM sulfoxaflor would be capable of releasing DA from the TIDA neurones. Together, the data support the hypothesis that through its central nAChR agonist properties, sulfoxaflor increases DA efflux from TIDA neurones in the median eminence, and in turn, this effect is predicted to result in a decrease of prolactin secretion from the pituitary gland in the rat.

In summary, sulfoxaflor caused concentration related increases in local dopamine concentrations possibly because of increased release or enhancement of synaptic longevity due to slower synaptic reuptake or both, from the mediobasal hypothalamic dopaminergic neurones when reverse dialysed into this brain region. The effects are potentially of pharmacological and physiological relevance. The data support the hypothesis that sulfoxaflor may increase dopamine efflux from TIDA neurones in the median eminence and that this effect would be predicted to result in a decrease of prolactin secretion by the anterior pituitary gland.

Study 9: MoA Study: Screening for Oestrogen Receptor and Androgen Receptor Binding and Transactivation and Aromatase Inhibition. DAR Section B.6.5.4.3.

Report: Toole, C. (2011). XDE-208 Technical: Screening for Estrogen Receptor and Androgen Receptor Binding and Transactivation and Aromatase Inhibition. CeeTox, Inc. 4717 Campus Drive, Kalamazoo, Michigan, USA. Unpublished.

Report No.: DR-0404-3134-123; Report Number: 9115-100297.

Dates: 2011

Guidelines: Non-guideline.

GLP: No. Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were not provided.

Deviations: This is an acceptable though non-guideline study, it is considered supplementary to the long-term chronic / carcinogenicity studies and suitable for a MoA investigation.

Deficiencies: No.

Executive Summary: These studies describe the ability of sulfoxaflor to interact with the oestrogen and androgen receptors and inhibit aromatase activity. Sulfoxaflor is identified as a

non-binder in the oestrogen receptor alpha (ERα) fluorescence polarisation (FP) assay as no displacement of the fluormone from the oestrogen receptor occurred. Sulfoxaflor is categorised as a potential binder in the androgen receptor (AR) FP assay as the binding curve exceeded the required 50% displacement of the fluormone from the receptor. The AR and ER transactivation assays were negative for agonism or antagonism by sulfoxaflor. Based on this, sulfoxaflor-related non-specific interaction cannot be ruled out as a potential mechanism of action for the response observed in the AR-binding assay, as no biological effect was identified in an AR-mediated transactivation assay. The aromatase assay determined that sulfoxaflor did not inhibit aromatase (CYP19) activity. The results from the five different *in vitro* screening tests with sulfoxaflor described further did not indicate changes consistent with endocrine-mediated alterations.

Sulfoxaflor was assessed in 5 different assays in order to determine its potential for endocrine activity. The assays performed were as follows: ERα binding (FP), AR binding (FP), ER and AR transactivation (agonism and antagonism), and AR aromatase inhibition. The top concentration of sulfoxaflor for use in the assays was 10^{-3}M. Two independent runs of each assay were performed. Sulfoxaflor did not demonstrate any agonism or antagonism in the ER and AR transactivation assays. Reference controls demonstrated that the systems were performing as expected and able to detect mild agonism and antagonism for both ER and AR.

The aromatase assay determined that sulfoxaflor did not inhibit aromatase (CYP19) activity. Overall, the results from these five different *in vitro* screening tests with sulfoxaflor did not indicate changes consistent with sex steroid or classical endocrine-mediated alterations.

Study 10: Human Relevance Framework for Leydig cell Tumours. (DAR Section B.6.5.4.4.)

Report: R. J. Rasoulpour, C. Terry, M. J. LeBaron, R. G. Ellis-Hutchings, and B. B. Gollapudi (2011). Compound: XDE-208 (Sulfoxaflor): Mode Of Action And Human Relevance Framework Analysis For XDE-208-Induced Promotion Of Fischer 344 Rat Leydig Cell Tumors. Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan, 48674. Unpublished.

Report No.: DR-0404-3134-122; Study ID: 110101.

Dates: 2011

Guidelines: Non-guideline. Not required for EU dossier submission. It is however a useful substance summary of the data regarding sulfoxaflor exposure and Leydig cell tumour incidence and relevance to man. This is submitted as a supplementary study/assessment in support of this DAR.

GLP: Not applicable.

Deviations: None. This is an acceptable overview of all the data presented thus far in section B6.5 as pertains to sulfoxaflor-induced Leydig cell tumours in rodents and the toxicological relevancy of this effect to man.

Deficiencies: None. General discussion document.

Abstract: Sulfoxaflor caused an increased size of Leydig cell tumours (LCT) at 100 and 500 ppm in a Fischer F344 Du/Crl rat carcinogenicity study. Histopathological examination

confirmed that there was no increase in the overall incidence of LCT across the groups with 88, 92, 90, and 92% of rats affected at 0, 25, 100, and 500 ppm, respectively. However, there was a significant increase in bilateral LCT incidence at 500 ppm (88%) when compared to controls (64%). The background incidence of Fischer rat LCT is 75-100% in 2-year studies (88% for controls in the sulfoxaflor study) compared to 1-5% in CD rats, even less in CD-1 mice, and orders of magnitude lower in ranges of 0.01 – 0.00004% for humans. These interspecies differences in background incidence are well understood, and are the result of quantitative and qualitative differences of Leydig cell response to hormonal stimuli. Rat Leydig cells contain > 10-fold more luteinizing hormone (LH) receptors than humans, which confers greater sensitivity to slight changes in circulating LH levels. In addition to this quantitative difference, rat, but not human, Leydig cells express both prolactin receptors and gonadotropin releasing hormone (GnRH) receptors on their surface. Stimulation of rat Leydig cells through both prolactin and GnRH receptors are a rat-specific mechanism by which LCT formation can occur. For prolactin receptor involvement in LCT, dopamine agonists (e.g., pharmaceutical class of drugs including bromocriptine) reduce prolactin release by the anterior pituitary gland eventually resulting in sustained elevations in pituitary LH release and Leydig cell stimulation and hyperplasia over a chronic duration.

Given these differences between rat and human Leydig cells, independent experts have determined "*…that human Leydig cells are quantitatively less sensitive than rat Leydig cells in their proliferative response to LH, and hence in their sensitivity to chemically induced LCTs. It can be concluded that no observable effect levels (NOELs) for the induction of LCTs in rodent bioassays provide an adequate margin of safety for protection of human health and that the data support a nonlinear mode of action (i.e., threshold response).*" Finally these experts conclude that "*...the data suggest that nongenotoxic compounds that induce LCTs in rats most likely have low relevance to humans under most exposure conditions because humans are quantitatively less sensitive than rats.*"

Analysis of the comprehensive array of available toxicology data for sulfoxaflor, including extensive non-cancer mode-of-action (MoA) data suggested a hormone-based dopamine enhancement MoA as the most likely cause of the LCT effect, which would operate through the the following key events: 1) increased neuronal dopamine release via specific dopaminergic neuron-based nicotinic acetylcholine receptor (nAChR) agonism, leading to 2) decreased serum prolactin levels, leading to 3) downregulation of LH receptor gene expression in Leydig cells, leading to 4) transient decreases in serum testosterone (T), leading to 5) increased serum LH levels, leading to 6) promotion of Leydig cell tumourigenesis. This hypothesis was evaluated in a specific MoA study in which these key events were examined to determine the causality of sulfoxaflor's promotion of Fischer rat LCT in the oncogenicity study. Additional studies were also conducted to examine whether other known potential MoAs were involved in the LCT promotion effect of sulfoxaflor. This document represents the weight of evidence approach used to evaluate the data based upon the Bradford-Hill criteria followed by subsequent application in a Human Relevance Framework (HRF).

The conclusion from this evaluation is that the LCT promotion observed in the oncogenicity study was through a subtle, but chronic, dopamine enhancement MoA in a uniquely susceptible animal model, the Fischer 344 rat. The data for sulfoxaflor are judged with a moderate degree of confidence to adequately explain the promotion of Fischer rat Leydig cell tumours following chronic dietary administration of sulfoxaflor, and judged with a very high degree of confidence to support a hormonally-mediated, threshold based, nonlinear MoA.

The promotion of Fischer rat LCT observed in the oncogenicity study has an MoA that is

hormonally-mediated and threshold-based, and should be considered to have no relevance to humans due to qualitative and quantitative differences between rat and human Leydig cells. On this basis, the Fischer 344 rat Leydig cell tumours associated with lifetime administration of high dose levels of sulfoxaflor would not pose a cancer hazard to humans.

Conclusions:

Statement of confidence in the evaluation. This MoA and Human Relevance Framework evaluation for sulfoxaflor-induced Leydig cell tumours in Fischer rats follows the guideline established for this process (*Sonich-Mullin et al., 2001; Cohen et al., 2003; Meek et al., 2003; USEPA, 2005; Boobis et al., 2007*). The extensive toxicological database for sulfoxaflor, including several focused *in vitro* and *in vivo* MoA experiments are high quality studies, which provide the necessary data to evaluate the MoA for sulfoxaflor-induced rodent Leydig cell tumours. Analysis of these data revealed a proposed hormone-based dopamine enhancement mode-of-action (MoA) through the following key events: 1) increased neuronal dopamine release via nicotinic acetylcholine receptor (nAChR) agonism, leading to 2) decreased serum prolactin levels, leading to 3) downregulation of luteinizing hormone (LH) receptor gene expression in Leydig cells, leading to 4) transient decreases in serum testosterone, leading to 5) increased serum LH levels, leading to 6) promotion of Leydig cell tumourigenesis. The subtle nature of the supportive data for this MoA is not surprising given the latency and subtle nature of the effects in question. The two findings that anchor the analysis to the dopamine enhancement MoA are the decreased serum prolactin levels and concomitant decrease in LHR gene expression. These findings are unique to the key event progression of this particular MoA.

The conclusion from this evaluation is that the LCT promotion observed in the oncogenicity study was through a subtle, but chronic, dopamine enhancement MoA in a uniquely susceptible animal model, the Fischer 344 rat. The data for sulfoxaflor are judged with a moderate degree of confidence to adequately explain the promotion of Fischer rat Leydig cell tumours following chronic dietary administration of sulfoxaflor, and judged with a very high degree of confidence to support a hormonally-mediated, threshold based, nonlinear MoA.

Other possible MoAs for Leydig cell tumourigenesis as described (*Cook et al., 1999*) have been evaluated with respect to sulfoxaflor. This in-depth analysis of alternative MoAs revealed direct and/or indirect data to refute the eight other known possible MoAs to develop rodent LCTs. Importantly, very strong *in vitro* and *in vivo* data exist to refute a genotoxic mechanism. Taken together, all other MoAs have been dismissed for sulfoxaflor induced LCT because they lack plausibility and coherence with the significant data from the mechanistic and guideline toxicity studies on sulfoxaflor.

Identification of data gaps. Due to the subtle nature and long latency for the effects in question, in combination with feedback compensation by the HPG axis, it is not surprising that the hormone and associated key events are transient during short-term studies. Therefore, these are not considered data gaps as it is more a function of the underlying biology. However, there are three data gaps identified during the analysis of this MoA, which are 1) lack of direct data for Key Event #1, 2) lack of direct data for Key Event #4, and 3) incomplete demonstration of key events at the 100ppm dose level.

Key Event #1 within this MoA is increased dopamine release via agonism on central dopaminergic neurons nAChRs. As outlined within the analysis of this key event, due to a combination of limited characterisation of nAChRs within the median eminence and technical

and biological complexity of measuring neurotransmitters within the hypothalamic-hypophyseal portal vein system, there are no direct data supporting Key Event #1. However, as there is a direct inverse correlation between prolactin and dopamine, the decrease in serum prolactin levels within Key Event #2 can be used as indirect support for Key Event #1. Results from the *in vivo* dopamine microdialysis study indicate that sulfoxaflor may increase extracellular dopamine levels in the mediobasal hypothalamus, an area near to the median eminence and acting as a surrogate target to the actual presumed target of sulfoxaflor *in vivo* – the tuberoinfundibular (TIDA) system (section B.6.5.4.2; *Rowley & Heal, 2011*).

Key Event #4 within this MoA is a transient decrease in serum testosterone levels. Under the conditions of the LCT MoA study, there were no measurable decreases in serum testosterone; however, as described within the analysis of Key Event #4, the delay in balanopreputial separation from the two-generation reproductive toxicity study supports a transient decrease in testosterone. While these data are supportive and provide strong indirect evidence on a testosterone effect, there are no hormone measurement data that show a decrease in serum levels of testosterone.

Finally, while there are data supporting the MoA at 500ppm, no precursor key events were observed at 100ppm. A dose-response relationship for these apical end point effects existed with increased testis size and increased incidence of bilateral tumours at 500ppm. Due to the high background incidence of these tumours in Fischer rats, the lack of precursor key events for this subtle, hormone-based MoA at the lower 100ppm dose level is not surprising, especially given the transient and compensatory nature of hormone regulation in the HPG axis

Implications for risk assessment. Sulfoxaflor causes promotion of Leydig cell tumours (LCT) in a Fischer rat carcinogenicity study. The effect in question is subtle in nature and the background incidence of Fischer rat LCT is 75-100% in 2-year studies compared to 1-5% in CD rats, even less in CD-1 mice, and orders of magnitude lower in ranges of 0.01 – 0.00004% for humans. These interspecies differences in background incidence are well understood, and result from quantitative and qualitative differences of Leydig cell response to hormonal stimuli. Rat Leydig cells contain >10-fold more LH receptors than humans, which confers greater sensitivity to slight changes in LH levels. In addition to this quantitative difference, rat, but not human, Leydig cells have both PrlR and GnRH receptors (GnRHR) on their surface. Stimulation of rat Leydig cells through both PrlR and GnRHR are a rat-specific mechanism by which LCT formation can occur. For PrlR involvement in LCT, dopamine agonists (e.g., muselergine) reduce Prl release by the anterior pituitary gland. This results in decreased binding of Prl to PrlR on Leydig cells, leading to downregulation of the LH receptor and transient reductions in testosterone production, which feeds back to induce LH release from the pituitary leading to Leydig cell stimulation and hyperplasia over time.

Given these differences between rat and human Leydig cells, independent experts have determined that "*that human Leydig cells are quantitatively less sensitive than rat Leydig cells in their proliferative response to LH, and hence in their sensitivity to chemically induced LCTs. It can be concluded that no observable effect levels for the induction of LCTs in rodent bioassays provide an adequate margin of safety for protection of human health and that the data support a nonlinear mode of action (i.e., threshold response).*" Finally the authors conclude that "*the data suggest that nongenotoxic compounds that induce LCTs in rats most likely have low relevance to humans under most exposure conditions because humans are quantitatively less sensitive than rats*".

Taken together, the promotion of Fischer rat LCT observed in the oncogenicity study has a MoA that is hormonally-mediated and threshold-based, and would be considered to have no relevance to humans due to qualitative and quantitative differences between rat and human Leydig cells. On this basis, the Fischer rat Leydig cell tumours associated with administration of high dose level of sulfoxaflor would not pose a cancer hazard to humans. Based on this hazard assessment for the sulfoxaflor-induced LCT effect, a margin of exposure risk assessment based on the chronic reference dose (cRfD) would be protective of human health.

4.10.3.3. Mechanism of action studies to address Prepucial Gland Carcinoma in rodents.

Study 11: Human Relevance Framework for Preputial Gland Carcinoma. DAR Section B.6.5.4.5.

Report: K. E. Stebbins, R. J. Rasoulpour and K. Boekelheide. (2011). XDE-208 (sulfoxaflor): mode of action and human relevance framework analysis of preputial gland carcinomas in the two-year f344/ducrl rat carcinogenicity assay. Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan, 48674. Unpublished.

Report No.: Study ID: 110175.

Dates: 2011

Guidelines: Non-guideline. Not required for EU dossier submission. It is however a useful summary of the data regarding sulfoxaflor exposure and preputial gland tumour incidence and relevance to man. This is submitted as a supplementary study/assessment in support of this DAR.

GLP: Not applicable.

Deviations: None. This is an acceptable overview of all the data presented thus far in section B6.5 as pertains to sulfoxaflor-induced preputial gland tumours in rodents and the toxicological relevancy of this effect to man. There is quite a bit of overlap with the information contained within section B.6.5.4.4. As in many other sections of this DAR, a reference list is compiled at the end of each subsection relating to the peer reviewed literature for the endocrine effects thought to be responsible for the mode of action.

Deficiencies: None. General discussion document.

Abstract: Sulfoxaflor caused a marginal increased incidence of preputial gland carcinoma, which did not reach statistical significance, in the F344/DuCrl rat carcinogenicity study. This effect was limited to the high dose level of 500ppm, with a no-observed-effect level of 100ppm (4.24 mg/kg bw/ day). The proposed mode-of-action (MoA) for this effect includes the following Key Events (KE), and is not relevant to humans:

- Agonism, via nicotinic acetylcholine receptors, to dopaminergic neurons in the hypothalamus resulting in increased dopamine release.

- Dopamine-mediated inhibition of prolactin release from the anterior pituitary resulting in reduced serum prolactin levels.
- Reduced stimulation of prolactin receptors on Leydig cells resulting in reduced luteinizing hormone (LH) receptor density on Leydig cells (human Leydig cells do not have functional prolactin receptors and hence the sequence of events beyond this step cannot occur in humans).
- Reduced LH receptor density leads to transiently reduced testosterone production by Leydig cells.
- Reduced serum testosterone levels stimulates increased production of LH from the pituitary
- The continuous drive of increased dopamine release leads to a 'resetting' of the hypothalamic-pituitary-gonadal (HPG) axis to a slightly higher level of activity and hence higher testosterone production.
- The slightly higher testosterone level stimulates preputial gland proliferation which, over a lifetime, promotes normal spontaneous tumourigenesis in the rat preputial gland.

Table 4.10.3.1.Study 11.1 (DAR Table 6.5.4.5-1: Sulfoxaflor): Temporality and dose response for MoA key events related to male F344/DuCrl rat preputial gland carcinoma.

Temporal →

Dose ↓

Dose (ppm)	Key Event 1 Increased dopamine release via nAChR agonism	Key Event 2 Decreased serum prolactin levels	Key Event 3 Downreg of LHR gene expression in Leydig cells	Key Event 4 Transient decreased serum testosterone levels	Key Event 5 Increased serum LH levels	Key Event 6 Reset of HPG axis / increased serum testosterone	Key Event 7 Promotion of preputial gland tumors
25		-	-	-	-		-
100		-	-	-	-		-
400				+*			
500	+	+	+	-	+	**	+
	+ indicates effect present, - indicates effect absent, blank cell indicates no data. * indicates indirect data from delay in balanopreputial separation data. **indicates no direct data, but supportive evidence in the literature.						

Overall, the weight of evidence (WoE) supports no relevance of preputial gland carcinomas for human health risk assessment because:

- The MoA for sulfoxaflor-induced preputial gland carcinoma is not relevant to humans.

- Sulfoxaflor has no indication of genotoxicity from *in vitro* and *in vivo* assays for mutagenicity or clastogenicity.
- Humans do not have an anatomic equivalent to rodent preputial glands.
- There were no effects in the female rat correlate to the preputial gland (clitoral gland).
- Even at higher doses, there were no effects in CD-1 mouse preputial glands, clitoral glands, or other sebaceous glands (skin, Zymbal's gland).
- There were no effects in other sebaceous glands (skin, Zymbal's gland) in male or female F344/DuCrl rats.

In summary, the MoA for the sulfoxaflor's promotion of preputial gland carcinoma is dopamine enhancement, which is the MoA responsible for the Leydig cell tumour promotion and its associated effects on the epididymides and accessory sex glands of F344/DuCrl rats. This is a hormonally-mediated, threshold based, nonlinear MoA. As indicated by published literature (*Cook et al., 1999*), this MoA is not relevant to humans.

4.10.4 Summary and discussion of carcinogenicity

Summary of long-term toxicity, carcinogenicity and mode of action studies:

A. Synopsis of the studies in the CLH report section 4.10.1-3: *(DAR Sections B.6.5.1.1 and B.6.5.2.1.*)

There were no clinical findings of significance due to active substance exposure and no dose related responses observed for the lifetime of the chronic studies in both rats and mice beyond geriatric diseases what would normally be expected from an ageing population. After 18 and 24 months there were no statistically significant differences in mortality between the study groups for either males or females at any dose level in mice and rats respectively. In general, there were no treatment-related differences in feed consumption throughout the duration of the studies; average feed consumption data for each group and each species was very similar to controls. Clinical pathology was unremarkable for all groups.

Sulfoxaflor's key target organs in the long-term toxicity studies were the liver in rats and mice and testes and preputial gland in male rats. Non-neoplastic treatment-related liver effects were as follows:

- Increased serum cholesterol levels at 3, 6, and 12 months in high dose level male rats given 21.3mg/kg/day and at 3, 6, 12 and 18 months in high dose level female rats given 39mg/kg/day;
- Increased liver weights in high dose level rats (at 12 months) given 21.3mg/kg/day (males) or 39mg/kg/day (females) and in high dose level mice given 79.6mg/kg/day (males) or 176mg/kg/day (females);
- Hypertrophy, fatty change and multifocal single cell necrosis of hepatocytes in high dose level rats given 21.3mg/kg/day (males) or 39mg/kg/day (females) and in high dose level mice given 79.6mg/kg/day (males) or 176mg/kg/day (females). In addition, aggregates of macrophages/histiocytes were increased in severity in high

dose level male and female rats and, in high dose level male mice, increased incidences of eosinophilic and vacuolated foci and 'hepatocytes in mitosis'.

- The only treatment-related finding in mid-dose level female mice given 33.9mg/kg/day was an increased incidence of slight hepatocellular hypertrophy. However, this finding was interpreted to be an adaptive, non-adverse response per se due to a complete lack of any associated changes including increase in liver weight or any treatment-related histopathological findings.

In addition to liver effects, high dose level male mice given 79.6mg/kg/day had an exacerbation in the cumulative incidence of spontaneous dermatitis, which is common in CD1 mice. This was interpreted to be secondary to general unthriftiness and stress induced by liver tumours in the high dose group.

Increased Leydig cell (interstitial cell) adenoma mass was seen in the testes of male F344 rats given sulfoxaflor at 4.24 or 21.3mg/kg/day (100 or 500 ppm). The larger Leydig cell adenomas resulted in higher testicular weights. The absolute testes weights of males given 100 or 500ppm were approximately 46% and 62% higher than controls, respectively. Secondary effects to the larger Leydig cell adenomas consisted of severe atrophy of testicular seminiferous tubules (100 and 500ppm), decreased amount of sperm in the epididymides (100 and 500ppm), decreased secretory material in the accessory sex glands (500ppm), and an increase in the incidence of preputial gland carcinomas (500ppm). With respect to the preputial gland tumours, a previous study recorded a similar association with a dose-related increase in preputial gland tumours suggested to be secondary to the disturbed endocrine balance of 3-monochloropropane-1,2-diol treated Fischer 344 rats with large Leydig cell adenomas (*Sunahara et. al., 1993*).

With respect to human risk assessment, the relevancy of these endpoints is questionable at best. There is much speculation over whether rat testicular Leydig cell tumours (LCT) have any relevance to human toxicology (*Prentice & Meikle, 1995; Clegg, et al, 1997; Cook et al, 1999*). It is the most frequently encountered neoplasm of the rat testis, the incidence of which varies greatly among strains. The rate increases with age but varies from 1 – 2% in Long-Evans rats, 1 – 5% in Sprague-Dawley rats, 4 – 7% in Wistar rats, 78% in Wistar substrain U rats to nearly 100% in Fischer 344 rats (*Turek and Desjardins, 1979; Boorman, et al, 1990; Teerds et al., 1991; Cook et al, 1999).* In contrast, the age-adjusted rate in humans has been reported to be only 0.4 per million (0.00004%) (*Gilliland & Key, 1995*). Differences between rat and human Leydig cells have been proposed that suggest that human Leydig cells are quantitatively and qualitatively less sensitive than rats in their responses to luteinising hormone (LH) and gonadotropin releasing hormone (GnRH), and hence in their sensitivity to chemically induced LCTs. Interestingly, several human epidemiology studies with a number of compounds that induce LCTs in rats (1,3-butadiene, cadmium, ethanol, lactose, nicotine) do not demonstrate any link between human exposure to these compounds and Leydig cell hyperplasia or tumours (*Cook et al., 1999*).

With respect to the preputial gland tumours, a previous study recorded a similar association with a dose-related increase in preputial gland tumours that was suggested to be secondary to the disturbed endocrine balance of 3-monochloropropane-1,2-diol treated Fischer 344 rats with large Leydig cell adenomas (*Sunahara et. al., 1993*).

Summary Table 4.10.4-1 (DAR Table 6.5.5.1-1): Summary of long term toxicity and carcinogenicity of Sulfoxaflor							
DAR Section	**Study**	**Species/ strain**	**Dosages**	**NOAEL**	**LOAEL**	**Target organ/ principal effects at LOAEL**	**Report ref. (study ID)**
B.6.5.1.1	2-year combined toxicity and carcino-genicity dietary	Rat/ F344	♂: 0, 25, 100, 500ppm equivalent to 0, 1.04, 4.24, and 21.3mg/kw bw/day respectively.	100ppm (4.24mg/kg bw/day)	500ppm (21.3mg/kg bw/day)	*12 month interim sacrifice and end of study:* Liver - increased blood cholesterol, liver weight, hypertrophy, fatty change, single cell necrosis and macrophages. *End of study:* Increased testes weight due to larger Leydig cell adenomas; secondary effects included atrophy of seminiferous tubules, reduced sperm in epididymides and secretory material in accessory sex glands. *High dose*: increased incidence and size of Leydig cell adenomas with secondary effects including preputial gland tumours; liver adenomas**	*Stebbins et al. 2010* (071187)
B.6.5.2.1	18-month carcino-genicity dietary	Mouse/ CD1	♂: 0, 25, 100, 750ppm equivalent to 0, 2.54, 10.4, 79.6mg/kg bw/day	100ppm (10.4mg/kg bw/day)	750ppm (79.6mg/kg bw/day)	Liver – adenomas and carcinomas**; Increased liver weight, hypertrophy with eosinophilia, fatty change, single cell necrosis, eosinophilic/ vacuolated foci, mitosis.	*Thomas et al. 2010b* (081102)

***Considered not relevant to humans*

Summary Table 4.10.4-2 (DAR Table 6.5.5.1-2): Summary of mode of action and supporting data				
DAR Section	**Species/ strain**	**Dosages (ppm)**	**Results**	**Report Ref. (study ID)**
Liver effects:				
B.6.5.3.1	Mouse/CD1 ♀)(and Rat/F344 (♂ and ♀)	Mice: 0, 3000, 4500. Rats: 0, 2000	Sulfoxaflor-induced gene expression profile in mice and liver (hepatocellular) proliferation in both mice and rats characteristic of phenobarbital-like CAR agonism.	*Geter & Kan 2008* (081102)
B.6.5.3.2	Rat/F344 (♂ and ♀)	0, 100, 750, 1500 for 3 or 7 days	Sulfoxaflor-induced liver effects were PB-like. Males were affected more than females. Neither AhR nor PPARα were involved.	*Geter & Card 2010* (070339)
B.6.5.3.3	Mouse/CD1 (♂ and ♀)	Males: 0, 500, 750. Females: 0, 1000, 1500 for 7 days	Sulfoxaflor -induced liver effects were consistent with CAR activation resulting in a PB-like MoA; males were more sensitive than females. Neither AhR nor PPARα were involved.	*Geter et al. 2010* (080246)
B.6.5.3.4	Mouse/C57Bl/6J WT	0, 750, 1500	Sulfoxaflor -induced liver effects in C57Bl/6J WT mice were similar to previously observed effects in CD1 mice	*Elcombe 2010*
B.6.5.3.5	Mouse/C57Bl/6J WT, Humanised and KO PXR/CAR	0, 750 for 7 days	In WT C57BL/6J sulfoxaflor caused the same liver effects as seen in CD1 mice. In PXR/CAR KO mice, sulfoxaflor did not induce any liver changes, demonstrating that activation of one or both of these receptors is required to elicit the liver effects seen in WT mice. In PXR/CAR humanised mice slight liver hypertrophic effects occurred but not hepatocellular proliferation. This study demonstrated that sulfoxaflor, like PB, acts via a CAR-mediated MoA and that mice carrying the human PXR and CAR receptors did not develop hepatocellular proliferation responsible for liver tumour induction. Therefore, sulfoxaflor -induced rodent liver tumours are not relevant to humans.	*Ross 2010* (100125)
B.6.5.3.6	Human Relevance Framework for Liver Tumours		Sulfoxaflor -induced rodent liver tumours occur via a CAR-mediated MoA for which there is a high level of confidence. There is no evidence of increased hepatocellular proliferation in humanised mice treated with sulfoxaflor or in humans exposed to	*LeBaron et al., 2010* (100291)

Summary Table 4.10.4-2 (DAR Table 6.5.5.1-2): Summary of mode of action and supporting data				
DAR Section	**Species/ strain**	**Dosages (ppm)**	**Results**	**Report Ref. (study ID)**
			high doses of phenobarbital (PB). A hepatocarcinogenic response in rodents for compounds which have data to support a PB-like MoA is considered not relevant to humans. On this basis, the rodent liver tumours associated with administration of high dose levels of sulfoxaflor would not pose a cancer hazard to humans.	
Leydig cell effects				
B.6.5.4.1	Rat/F344 and Crl:CD(SD) (♂); testosterone elimination and dopamine agonism and / or enhancement MoA study.	0, 25, 100, 500	Support dopamine enhancement MoA for LCT promotion: ↓ Prl levels at 4-wks, ~2-fold dose-dependent ↓ LHR gene expression at 4-wks, ↓ PrlR gene expression at 4-wks.	*Rasoulpour, 2010* (101105)
B.6.5.4.3	- hERα AR ligand binding domain - T47D-KBluc cell line (ER) - MDA-kb2 cell line (AR) - Recombinant microsomes	0-1.0 mM	Negative for ER binding. Negative for ER and AR transactivation assays (agonism and antagonism). Negative for aromatase (CYP19) inhibition.	*Toole, 2011* (110030)
B.6.5.4.4	Human Relevance Framework for Leydig cell Tumours		Sulfoxaflor -induced promotion of LCT occurs via a subtle, but chronic, dopamine enhancement MoA in a uniquely susceptible animal model, the Fischer 344 rat. The data for sulfoxaflor are judged with a moderate degree of confidence to adequately explain the promotion of Fischer rat Leydig cell tumours following chronic dietary administration of sulfoxaflor, and judged with a very high degree of confidence to support a hormonally-mediated, threshold based, nonlinear MoA.	*Rasoulpour et al., 2011* (110101)
B.6.5.4.2	Dopamine microdialysis experiment		Sulfoxaflor (400µM and 2mM) produced concentration related increases in the extracellular level of dopamine in the mediobasal hypothalamus. The results indicate that sulfoxaflor causes a firing dependent increase of dopamine exocytosis from hypothalamic dopaminergic neurones. The data support the hypothesis that through its nAChR partial agonist properties	*Rowley and Heal* (2011)

Summary Table 4.10.4-2 (DAR Table 6.5.5.1-2): Summary of mode of action and supporting data				
DAR Section	**Species/ strain**	**Dosages (ppm)**	**Results**	**Report Ref. (study ID)**
			sulfoxaflor increases dopamine efflux from TIDA neurones in the median eminence, and in turn, this effect is predicted to result in a decrease of prolactin secretion from the pituitary gland in the rat.	
B.6.5.4.5	Human Relevance Framework for Preputial Gland Carcinoma		The MoA for sulfoxaflor's promotion of preputial gland carcinoma is dopamine enhancement, which is the MoA responsible for the Leydig cell tumour promotion and its associated effects on the epididymides and accessory sex glands of F344/DuCrl rats. This is a hormonally-mediated, threshold based, nonlinear MoA. This MoA is not relevant to humans.	*Stebbins et al. (2011)*

B. Evidence for carcinogenicity:

Rat: Dietary administration of sulfoxaflor resulted in tumours of the liver, testes and the preputial glands in male rats. There was no evidence of carcinogenicity in female rats.

(1) Liver Tumours: In male rats, statistically significant trends ($p<0.01$) were seen for both hepatocellular adenomas and the combined tumour types (adenomas/carcinomas). When compared to controls, a statistically significant increase in pairwise comparison was seen for hepatocellular adenomas ($p<0.01$) and combined adenomas/carcinomas ($p<0.05$) at the high dose (500ppm). The incidences of liver tumours at the high dose exceeded the testing laboratories historical control range. The liver tumours were corroborated by the presence of non-neoplastic lesions of the liver in male rats. No treatment-related liver tumours were seen in female rats. It is considered that the liver tumours in male rats to be treatment-related at 500ppm.

(2) Leydig Cell Tumours: There was a statistically significant increase in the incidence of bilateral, but not unilateral adenomas at the high dose when compared to both historical and concurrent controls. In addition, there was also a dose-response assocated with the occurrence of bilateral adenomas and testicular weight indicative of tumour load. However, when evaluating the combined incidences of this neoplasm (i.e., unilateral and bilateral), there was no dose response and no statistical significance. The incidences of the combined neoplasm (92%) were within the testing laboratories historical control range (76 – 92%) and the concurrent controls (88%). F344 rats are known to have high background rates for Leydig cell tumours but it is considered that the Leydig cell tumours in these male rats to be treatment-related in the sence that there is an exacerbation of the tumour load as unilateral adenoma incidences fall and bilateral incidences increase with concomitant effects on secondary sexual organs as a side effect of increasing tumour load.

(3) Preputial Gland Tumours: An increased incidence of carcinoma of the preputial gland was observed at the high dose. However, histopathological examination of the preputial gland was conducted only when triggered by the presence of a gross lesion [i.e., not all of the animals (50 animals/group) underwent histopathological examination]. Since the preputial glands were not histopathologically examined in all animals, it is difficult to acertain the exact incidence of this tumour. It is considered that the preputial gland tumours that were observed to be potentially treatment related but that there is insufficient data to confirm the true incidence and whether or not a dose response is observed. There was no data available regarding preputial gland proliferation. Preputial gland tumours are not commonly diagnosed in bioassay studies and the positive response may be considered an unusual finding. The regulatory guidelines do not require the preputial gland to be preserved for routine histopathological examination; thus, the tissues are not available for re-examination.

Mouse: Dietary administration of sulfoxaflor resulted in tumours of the liver only, there were statistically significant trends ($p<0.01$) for hepatocellular adenomas, carcinomas and combined adenomas and/or carcinomas. Male mice were much more susceptible to the development of adenomas and carcinomas than female mice. There were no effects on the testes and the preputial glands in male mice.

(1) Liver Tumours: In male mice, when compared to controls, there were significant increases in pair-wise comparisons for hepatocellular adenomas ($p < 0.05$), carcinomas ($p < 0.01$), and combined adenomas and/or carcinomas ($p < 0.01$) at the high dose (750ppm; 79.6 mg/kg bw/day). The incidences of adenomas, carcinomas and the combined tumours at the high

dose exceeded the testing laboratories historical control mean range. In female mice, there were statistically significant trends for hepatocellular carcinomas ($p < 0.01$) and combined adenomas and/or carcinomas ($p < 0.05$). No statistical significance was seen in pair-wise comparisons with the controls for any tumour type. There was an increase in the incidences of carcinomas at the high dose (1250ppm; 176 mg/kg bw/day). Although this increase did not reach statistical significance, the incidences exceeded the historical control range for this tumour type and the malignancy was corroborated with the presence of non-neoplastic lesions at this dose. Additionally, there were supportive non-neoplastic lesions in the liver of both sexes. It is considered that the liver tumours in male mice at 750ppm to be unequivocally treatment-related.

C. Human relevance of rodent carcinogenicity caused by sulfoxaflor:

Liver Tumours: It is considered that the proposed mode of action (MOA) for the generation of liver tumours is plausible considering the data submitted. A MOA based on constitutive androstane receptor (CAR) activation was supported by the observation of increased Cyp2b enzyme expression and activation, increased liver weight, increased hepatocellular hypertrophy, and hepatocellular proliferation in both mice and rats. However, the use of the combined CAR/PXR knockout and hCAR/hPXR knockin mouse models does not delineate between CAR and PXR activities even though traditionally *Cyp2b* activity is primarily associated with activated CAR-mediated induction and *Cyp3a* activity is primarily associated with activated PXR-mediated induction. Significant overlap in the respective nuclear receptors ability to bind to DNA motifs and enhancer elements located in the regulatory regions and promoter sequences of either gene occurs and this has not been investigated in any detail. Nor has there been any investigation into the use of known species specific CAR/PXR activators with the transgenic mouse models employed (e.g. TCPOBOP for mouse CAR, 2,4,6-triphenyldioxane-1,3 – TPD for rat CAR, CITCO for human CAR, rifampicin for human PXR, pregnenolone-16α-carbonitrile – PCN for rat and mouse PXR). This would have helped to further strengthen the arguement for species specific CAR activity. The above noted effects are considered precursor events to liver tumour formation following a phenobarbital-like MOA, and such a MOA is not considered relevant to tumour formation in humans. Further, the observation of increased cell proliferation in wild type mice and the lack of a similar observation in CAR/PXR knockout and humanised mice is indicative of the specificity of the mouse CAR/PXR receptors' role in inducing the necessary precursor event of cell proliferation. The observation of all precursor key events was assessed at the turmourigenic dose in mice. Cytochrome 2b enzyme induction and expression and cell proliferation was only assessed (and observed) at a dose level above the tumourigenic dose in rats (750 ppm vs. 500 ppm). Nonetheless, all precursor events have general temporal and dose concordance with the observation of liver tumours.

Limited liver cytotoxicity by way of increased incidences of individual hepatic cell necrosis, scored as very slight in nature and observed in a number of studies may be correctly described as treatment related effects (90-day dietary studies in the rat and mouse, single-cell hepatocyte necrosis was observed at≥750 ppm (47 .6 and 98 mg/kg bw/day); 2-generation reproduction study, very slight centrilobular single cell necrosis of the liver in parental male Sprague-Dawley rats at the high dose of 400 ppm (24.6 mg/kg bw/day); in a mode of action study investigating liver weight effects in CD-1 mice, single cell necrosis was observed in males at 500 ppm (89 mg/kg bw/day) and above). However, these effects were generally seen at the tumourigenic dose in both rats and mice≥ 500ppm). Though initially this observation may not be consistent with a phenobarbital-like MoA, there was no evidence for extensive liver cytotoxicity from other histological indices or clinical chemistry. There were no significant

elevations in plasma hepatic transaminases to warrant concern for cytotoxicity as a major *modus operandi* for liver tumour development. It is not considered that the present available evidence is sufficient to suggest that sulfoxaflor may operate via more than one primary mode of action to induce liver tumours in rodents. Both activation of the CAR as well as some limited induction of cytotoxicity in the liver are occurring concordant with liver tumours but the weight of evidence from all the studies would suggest the primary activity is CAR/PXR activation followed by liver enzyme induction, hepatocyte proliferation with subsequent induction of proliferative lesions in the rodent liver including foci, adenomas, and carcinomas. Initial short term events such as CAR-dependent enzyme induction, liver weight increases and hepatocyte proliferation differ depending on the genetic constitution of the CAR/PXR nuclear receptors in mouse transgenic models and support the hypothesis that species-specific CAR activation is the probable cause of the liver tumours observed in the rodent studies at high concentrations of sulfoxaflor. In addition, there is no concern for mutagenicity. Neither sulfoxaflor nor its metabolites caused gene mutations or chromosome aberrations in *in vivo* or *in vitro* studies. In conclusion, the evidence supports a non-genotoxic, threshold based, mitogenic response similar to a phenobarbital (PB) like MoA for these rodent liver tumours.

Leydig Cell Tumours: Even though the background incidence of Leydig cell tumours is incredibly high in the Fisher 344 strain of rat, the opinion is that there is sufficient data in the longterm / carcinogenicity study that sulfoxaflor has a treatment related effect on the Leydig cell tumours observed at the end of the combined chronic/carcinogenicity study in F344 rats. There are clear indications of greater tumour burden with increased testicular weights, extensive secondary effects due to tumour mass and increased bilateral incidences.

In the rat, focal Leydig cell hyperplasia and Leydig cell tumours can be readily induced by a wide range of chemically diverse drugs and chemicals, including dopamine agonists, antiandrogens, LHRH analogs, peroxisome proliferators, and histamine receptor antagonists. The effect on rat Leydig cells observed in the 2-year carcinogenicity study is subtle in nature and confounded by the background incidence of Fischer 344 rat LCT which is 75-100% in 2-year studies compared to 1-5% in CD rats, even less in CD-1 mice, and orders of magnitude lower (0.01 – 0.00004%) for humans. These interspecies differences in background incidence are well known, and are thought to result from the quantitative and qualitative differences of Leydig cell responses to hormonal stimuli. Initially, a proliferative response in the F344 rat results in Leydig cell hyperplasia that, with chronic stimulation, may grow to form a LCT, typically a benign adenoma.

Rats are more prone than humans are to LCT because their Leydig cells may have more than ten times the quantity of LH receptors, which may impart a greater sensitivity to slight changes in circulating LH levels (*Huhtaniemi, 1983; Katzung, 1995*). In addition to this quantitative difference, rat, but not human, Leydig cells have both prolactin receptor (PrlR) and gonadotropin- releasing hormone receptors (GnRHR) on their surface (*Clayton and Huhtaniemi, 1982; Cook et al., 1999*). Stimulation of rat Leydig cells through both PrlR and GnRHR are a rat-specific mechanism by which Leydig cell tumour formation can occur. For PrlR involvement in LCT, dopamine agonists (e.g., muselergine) reduce Prl release by the anterior pituitary gland. This results in decreased binding of Prl to PrlR on Leydig cells, leading to downregulation of the LH receptor. Decreased LHR gene expression results in slight but transitory decreases in testosterone production, which feeds back to the hypothalamus and pituitary gland to cause a compensatory increase in circulating LH to maintain testosterone at physiologic concentrations. This transitory decrease in testosterone may be responsible for the treatment-related delay in balanopreputial separation (BPS) for male offspring in the high-dose group in the two-generation reproductive toxicity study. As

with all hormone-based, threshold mechanisms of rodent Leydig cell tumourigenesis, the compensatory increase in LH levels leads to increased Leydig cell proliferation and tumours over time.

It is considered that the proposed mode of action (MOA) for the Leydig cell tumours is plausible considering all of the data submitted. A MOA based on weak but sustained secondary dopamine release based on agonism of central nicotinic acetylcholine receptors on the cell bodies of the tuberoinfundibular (TIDA) neurones in the arcuate nucleus of the hypothalamus is considered plausible. A slight increase in dopamine concentration in the hypothalamic-hypophysial portal vessels would impact on the lactotrophs in the anterior lobe of the pituitary gland by further inhibiting prolactin secretion. Downstream consequences of reduced plasma prolactin would appear to be species specific to the rat (and mouse?) due to distinct molecular differences between rat and human Leydig cells. Publicised literature has well documented cases of Leydig cell tumours in rats upon treatment with dopamine agonists, there is little to no information to suggest that humans on dopamine agonist treatment are susceptible to an increased incidence of testicular tumours though there are perturbations in plasma testosterone response to hCG challenge (*Oseko et al., 1991*). Additionally, *Oseko* and colleagues showed that there were no significant changes to plasma LH in human males treated with bromocryptine while there were significant reductions in plasma prolactin (*Oseko et al., 1993*).

There are however uncertainties and inconsistencies in the results from the various studies:

- The postulated MoA includes decreased testosterone as a key event. There was a delay in preputial separation noted in males in the two-generation reproduction study that indicates a possible decrease in testosterone. However, no measurable decreases in serum testosterone were seen in the Leydig cell tumour MoA study.

- Although there were changes present related to specific key events (decreased prolactin and LH receptor expression), the changes observed were subtle and presented a weak dose response. Additionally, the only statistically significant changes were seen in dopamine release and LH levels and the LH changes were only seen at the tumourigenic dose.

- The concentrations used to evaluate dopamine release were based on the plasma concentration of rats after 12 months; therefore, it is unclear whether the concentrations are reflective of plasma concentration in rats after 24 months.

- No Leydig cell hyperplasia or proliferation was observed after sulfoxaflor exposure.

- Dopamine agonist positive controls would have helped in the interpretation of the results in some of the MoA studies.

Overall, the weight of evidence for the Leydig cell MoA suggests Sulfoxaflor causes further promotion of Leydig cell tumours (LCT) in the Fischer male rat. In conjunction with external evidence it would appear that interspecies differences in the background incidence of LCTs are well understood, and result from quantitative and qualitative differences of Leydig cell response to hormonal stimuli. Consequently it is considered that the MoA presented for sulfoxaflor has no relevance to humans and that sulfoxaflor is unlikely to pose a cancer hazard to humans.

Preputial Tumours: There was no direct experimental investigation into the effects causing preputial gland tumours. While there is insufficient data to explain the observed preputial tumours, or derive a mode of action, the available data was also inadequate to draw confident conclusions due to the small sample size and lack of histopathology data on all animals. Questions remain as to the actual incidence of this tumour type because only animals with palpatable masses were histologically evaluated in the long term carcinogenicity study so that the results cannot be interpreted as a proportion of the total number of animals in each treatment group. There is insufficent evidence regarding this effect but because humans do not have a preputial gland or equivalent, this finding in rats may have no relevance to humans, *per se*. The conclusion from the framework analysis was that the observed sulfoxaflor-induced promotion of preputial gland tumours is considered likely to be secondary to the LCTs, and thus of little human relevance. It was postulated that the effect is a consequence of resetting the HPG axis to a slightly higer level of activity resulting in a chronic increase in testosterone production.

The rat preputial gland is testosterone dependent for both its proliferation and differentiation (*Miyake et al., 1994; Ponmanickam et al., 2010*). While *Miyake et al., (1994)* make the point that androgen receptor mRNA is most abundant in the mid-differentiation sebocytes, rather than the less differentiated and more proliferative precursor cells, it is clear from several studies that testosterone provides a key proliferative signal to the rat preputial gland (*Freinkel, 1963; Ponmanickam et al., 2010*). Data to support an increase in serum testosterone due to resetting of the HPG axis mostly comes from the peer-reviewed literature with other dopamine agonists/enhancers.

Lowest Relevant Long-Term NOAEL*:* The liver tumours in high dose male F344 rats and male and female CD1 mice, and the increased size of LCT and their increased bilateral incidence, are not considered not relevant to humans. Similarly, preputial tumours are also not considered relevant. Therefore, the lowest relevant NOAEL from the long-term studies is:

4.24 mg/kg/day in male F344 rats based on based on decreased body weight gain, increased blood cholesterol and non-neoplastic liver effects (fatty change and single cell necrosis) in males at the next highest dose of 21.3 mg/kg/day.

D. Implications for Hazard Classification:

Rodent Liver tumours discussion:

Sulfoxaflor induced liver effects including the development of hepatocellular adenomas and/or carcinomas were, in general, similar to those induced by phenobarbital (PB), including the higher sensitivity of males to hepatocellular carcinogenesis than females (Jones et al. 2009). The mode of action (MoA) of PB induced liver tumours involves the activation of the constitutive androstane receptor (CAR), induction of cytochrome P450 enzymes, particularly *Cyp2b10* in mice, hepatocellular hypertrophy, increased hepatocellular proliferation, development of altered hepatic foci and ultimately, liver tumours (Holsappe et al. 2006). Recent studies investigating the MoA for sulfoxaflor -induced liver weight increases in mice showed significant elevation in *Cyp2b10* as well as *Cyp3a11*, CAR- and pregnane X receptor (PXR)-related genes, respectively in males and females. Benzyloxyresorufin (BROD) and Pentoxyresorufin (PROD) O-dealkylase liver enzyme activities which give a measure of *Cyp2b* enzyme induction were also elevated in male and female mice. Hepatocellular proliferation assessed by BrdU incorporation revealed elevated proliferation indices in sulfoxaflor treated males and females. These results support the hypothesis that sulfoxaflor -

induced liver effects are likely mediated through CAR in a similar manner as that of the prototypical CAR-agonist PB. The pivotal role of CAR in mediating PB induced liver tumours was reported by Yamamoto *et al.* 2004 wherein CAR knockout mice treated with PB failed to develop liver tumours. In order to determine if sulfoxaflor -induced liver effects were indeed mediated through CAR and thus a PB-like MoA, a confirmatory study was conducted with dual CAR/PXR knockout mice. Furthermore, to determine if human CAR/PXR were similar or different with regards to sulfoxaflor -induced liver effects, transgenic mice bearing humanized CAR/PXR were also used in this experiment (Ross, 2010) which tested the same dose that caused liver tumours in the sulfoxaflor mouse oncogenicity study (*i.e.*, 750 ppm). The results from this experiment showed that sulfoxaflor failed to induce liver effects such as liver weight increase, hepatocyte hypertrophy, hepatocellular proliferation, *Cyp2b10* and *Cyp3a11* expression, BROD and PROD enzyme activities in CAR/PXR knockout mice, consistent with observations reported by others with PB (Huang et al. 2005; Wei et al. 2000). However, while sulfoxaflor treated transgenic mice bearing humanized CAR/PXR had most of the aforementioned liver effects - which were qualitatively similar but weak CAR mediated events such as induction of *Cyp2b10* activity, protein and mRNA as well as liver weight increase compared to those of the wild type - the notable exception was treatment-related hepatocellular proliferation, which was totally absent. The studies reported here demonstrates that while human CAR/PXR mediated qualitatively similar CAR-mediated liver effects, albeit weaker as compared to those mediated by murine CAR/PXR, it did not mediate hepatocellular replicative DNA synthesis.

Since initial hepatocellular proliferation is the primary key event in a non-genotoxic MoA for the development of liver tumours (Holsapple et al. 2006) its absence in sulfoxaflor treated humanized CAR/PXR mice, supports the conclusion that sulfoxaflor would not be a human liver carcinogen (Ross, 2010) Furthermore, there are convincing data showing that human patients receiving PB for years, at doses producing plasma concentrations similar to those following carcinogenic doses in rodents, did not develop liver tumours. Taken together, on the basis of the robust data showing the MoA for sulfoxaflor mediated liver effects were PB-like, it is concluded that hepatocellular adenomas and/or carcinomas induced by sulfoxaflor would not occur in humans and thus, are not relevant for human risk assessment.

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Rat Leydig Cell Tumours discussion

The proposed sequence of events leading to effects on the epididymides and accessory sex glands is as follows:

1) Sulfoxaflor induces an increase in the size of testicular interstitial cell adenomas.
2) Large interstitial cell adenomas compress the seminiferous tubules and spermatogenesis is reduced.
3) Reduced spermatogenesis results in lower numbers of spermatic elements in the epididymides.
4) Reduced function of the testes results in lower amounts of secretory material produced by the accessory sex glands.

The higher incidence of preputial gland carcinomas in males at 500 ppm, relative to concurrent and historical controls, was also considered secondary to large interstitial cell adenomas of the testes. A similar association between the presence of large interstitial cell adenomas and increased incidence of preputial gland tumours was observed in a carcinogenicity study on 3-monochloropropane-1,2-diol in Fischer 344 rats (Sunahara, et al, 1993). In this previous study, a dose-related increase in preputial gland tumours was suggested to be secondary to the disturbed endocrine balance of treated animals with large interstitial cell adenomas. It should be noted that humans do not have an anatomical correlate to the preputial gland of rats (Monroe and Mordenti, 1995), and therefore, the higher incidence of preputial gland carcinomas in Fischer 344 rats has no relevance to humans, per se.

The toxicology of interstitial cell tumours and their relevance to humans have been reviewed extensively (Prentice, et al, 1995; Clegg, et al, 1997; Cook, et al, 1999). The initial alteration is hyperplasia of interstitial cells that can grow with age to the diameter of a single normal seminiferous tubule. When the proliferative interstitial cells reach a diameter of greater than a single normal seminiferous tubule, they are classified as adenomas, per guidance by the National Toxicology Program, NTP (Borman, et al, 1987 and1990).The high background incidence of interstitial cell tumours in Fischer 344 rats has been a well known phenomenon for decades with spontaneous adenomas even present at 12-months and increasing to 75-100% by 24 months (Turek and Desjardins, 1979). In contrast, the Sprague-Dawley rat has a

background incidence of 1-5% (Boorman, et al, 1990) at 24-months, while in the CD-1 mouse it is even lower at <1-2.5%. The molecular etiology behind why Fischer 344 rats have a unique predisposition to high spontaneous interstitial cell tumour incidence has not been fully elucidated. However, there are data that link the high rate of pituitary neoplasms (30.4% per National Toxicology Program (NTP) data (Haseman, et al, 1998); and disruption of the hypothalamus – pituitary – testis (HPT) axis with raising levels of serum and pituitary prolactin and estradiol levels and a concomitant decrease in follicle stimulating hormone (FSH) and luteinizing hormone (LH) in the aging Fischer 344 male rat (Amador et al, 1985; Bartke, et al, 1985). In addition, it is well documented that increased progesterone secretion occurs from interstitial cell tumours of Fischer 344 rats (Amador et al, 1985; Bartke, et al, 1985) with decreased secretion of testosterone and lower LH levels through negative feedback signaling (Gruenewald, et al, 1992)..

Fischer 344 rats are clearly predisposed to spontaneous interstitial cell tumours, with a lower prevalence in other strains of rat and lower still in mice. With regards to human relevance, estimates of human interstitial cell tumours are orders of magnitude lower with ranges of 0.01 – 0.00004% (Boorman, et al, 1990; Mati, et al, 2002) although there is a detection bias towards rats, as the testes of rats on chronic toxicity/oncogenicity studies are routinely examined microscopically, compared to human diagnoses based on palpable tumours that are confirmed by biopsy.

Despite strong similarities in the hypothalamic – pituitary – gonadal (HPG) axis among rats (Fischer 344 and Sprague-Dawley), mice, and humans, the stark difference in sensitivity to interstitial cell tumour formation implies significant differences must exist. The data support that these variations in background incidence may be primarily due to quantitative differences in interstitial cell response to stimuli via LH and gonadotropin- releasing hormone (GnRH) receptors.

In rodents and humans, LH stimulates interstitial cells to produce testosterone; however, rat interstitial cells have 20,000 LH receptors compared to only 1,500 in humans (Huhtaniemi, 1983). This greater than 10-fold higher number of LH receptors in the rat confers a greater sensitivity to slight changes in LH levels, compared to the relatively unresponsive human interstitial cell. It is due to the large number of extra receptors in the rat that LH receptor occupancy of only 1% is sufficient to elicit a single transduction cascade response, which confers the greater sensitivity in rats to slight changes in LH levels (Katzung, 1995)..

In addition to different LH receptor density, rat, but not human, interstitial cells have GnRH receptors (Clayton and Huhtaniemi , 1982) and prolactin receptors on their surface (Boorman, et al, 1990). Stimulation of rat interstitial cells through these receptors is a rat-specific mechanism by which interstitial cell tumour formation can also occur. For GnRH receptors, this position is supported by the fact that GnRH agonists such as buserelin can induce interstitial cell tumours and are purported to stimulate testosterone at low levels by direct activation at the interstitial cell but suppress testosterone through inhibition of LH release through negative feedback at the level of the pituitary gland, at higher doses. For prolactin receptor involvement in interstitial cell tumours, dopamine agonists, such as muselergine, reduce prolactin release by the anterior pituitary gland, which results in a decreased binding to prolactin receptors on interstitial cells (Prentice and Miekle, 1995). This decreased prolactin receptor stimulation results in downregulation of LH receptors and therefore lower testosterone levels, which feeds back to induce LH release from the pituitary leading to interstitial cell stimulation and hyperplasia (Prentice, et al, 1992).

As discussed here and reviewed extensively elsewhere, interstitial cell tumours in rats can be induced through alteration at the HPG axis resulting in excessive stimulation of interstitial

cells with Fischer 344 rats having an almost 100% spontaneous incidence of this tumour type (Boorman, et al, 1990; Clayton and Huhtaniemi , 1982) by 24 months of age. This is 10,000-1,000,000 times higher than published human incidences of this tumour type. Research into differences between rat and human interstitial cells supports this epidemiological data in the fact that rat interstitial cells are more responsive to perturbations in testosterone homeostasis due to a higher number of LH receptors and the presence of GnRH receptors on rat interstitial cell surfaces.

With regard to sulfoxaflor and the increased size, but not incidence of animals, with interstitial cell adenomas at 500 and 100 ppm in Fischer 344 rats, these findings are deemed to have low relevance to humans due to 1) the very high background incidence of this benign tumour in this strain of rat, 2) the absence of any endocrine-related effects at similar doses (high-dose level of 400 ppm) in the two-generation reproductive study, 3) the lack of these or related tumours in CD-1 mice and 4) the established data in the literature supporting quantitative differences in responsiveness of rat and human interstitial cells.

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4.10.5 Comparison with CLP and DSD classification criteria

4.10.5.1 Liver Tumours

DSD: (Directive 67/548/EEC)

Cat 1:

In accordance with the criteria in the DSD, classification in Category 1 for carcinogenicity is not justified as there is no evidence (epidemiological) of sulfoxaflor having caused cancer in humans.

Cat 2/Cat 3:

The adverse effects on liver tumour induction in the rat and mouse (2 species argument) would normally fulfil the criteria of Category 2 for classification for carcinogenicity. However, mechanistic evidence has been presented that the observed tumours in high dose animals are related to the activation of CAR/PXR nuclear receptors by sulfoxaflor and are species (rodent) specific. It has been proposed that this effect is not relevant to man due to functional differences in the CAR receptor between man and rodent species.

The criteria state that relevant arguments for a distinction between Cat 2 and 3 include *'..lack of genotoxicity in short-term tests in vivo and in vitro; existence of a species-specific mechanism of tumour formation irrelevant for man'*. Furthermore, for a distinction between Cat 3 and no classification the following argument is relevant where '*.. a substance should not be classified in any of the categories if the mechanism of experimental tumour formation is clearly identified,* ***with good evidence that this process cannot be extrapolated to man****'*.

Increased tumour incidences have been seen in two species so a simple argument for Category 2 classification could in theory be made. However, on consideration of all the available data, there are a number of factors that indicate classification in Category 2 is not appropriate. Sulfoxaflor has no structural relationship with other known carcinogens. There is a complete lack of genotoxicity seen with sulfoxaflor in *in vitro* and *in vivo* studies. The extensive mechanistic evidence has provided significant support for a phenobarbital-type, CAR-mediated mechanism to explain the liver responses and enzyme induction profiles in sulfoxaflor treated animals. The liver effects most often described involve an initial early event proliferation of hepatocytes, specific enzyme induction, liver hypertrophy with large increases in liver weight and activation of nuclear receptors; specifically CAR, often with some involvement of PXR. Transgenic mice humanised with respect to the CAR/PXR genotype show quantitatively and qualitatively different responses to the wild-type animals. Neither the knockout CARKO/PXRKO or hCAR/hPXR mice had increased hepatocellular proliferation. Gene transcription and enzyme induction assays confirmed a CAR/PXR mediated response and discounted involvement of other nuclear receptors such as the aryl hydrocarbon receptor and the PPARα receptor. Long-term treatments with PXR and CAR non-genotoxic activators such as the drug phenobarbital and the pesticide chlordane can

cause liver tumours in rodents, possibly due to their ability to increase cell proliferation and suppress apoptosis. While some general, non-regulatory data gaps exist such as no long term studies with transgenic animals, there is in balance, good evidence that the mechanism of liver tumour formation is not relevant for humans.

In view of these considerations, the available evidence is deemed to support a mechanism of action that is not relevant for human health (activation of CAR, induction of CYP isozymes, leading to increased hepatocellular proliferation with subsequent induction of proliferative lesions in the liver including foci, adenomas, and carcinomas). On the weight of the available evidence, classification for carcinogenicity is not proposed.

CLP:

Classification under the CLP regulation criteria is not proposed on the basis of the same findings. The CLP emphasises the importance of any substance-induced benign or malignant tumours in well formed animal studies as being potential indicators of human carcinogenic hazard with strength of evidence as a basis for classification into Categories 1A, 1B and 2 unless *'...there is strong evidence that the mechanism of tumour formation is not relevant for humans'*. Category 1A is precluded because of the lack of any human data with respect to sulfoxaflor exposure and carcinogenicity. Increased tumour incidences have been seen in two species so a simple argument for Category 1B classification could be made. However, good quality mechanistic data has been generated which constitute additional considerations that indicate classification in Category 1B is not appropriate because the liver responses parallel those common to rodent-specific non-genotoxic compounds such as phenobarbital and chlordane, involving activation of the nuclear receptors Constitutive Androstane Receptor (CAR) and Pregnane X Receptor (PXR). Additional mechanistic studies with humanised mice carrying the human CAR/PXR receptor genes instead of the wild type murine orthologs indicate that the human receptors do not support the proliferative effects seen in wild type mice with sulfoxaflor treatment (and seen with phenobarbital and chlordane in published studies using the same transgenic model). The weight of evidence supports the human non-relevance of the tumourigenic effect

4.10.5.2 Leydig Cell Tumours

CLP:

In accordance with the criteria in the CLP Regulation, classification in Category 1A for carcinogenicity is not justified as there is no evidence of sulfoxaflor having caused cancer in humans.

Increased Leydig cell tumour (LCT) incidences have been seen relative to concurrent controls in a single species only, the Fischer 344 rat. There was no evidence of such an effect in mice receiving higher doses of sulfoxaflor. There are a number of factors that indicate classification in Category 1B is not appropriate. Sulfoxaflor has no structural relationship with other known carcinogens. There is a complete lack of genotoxicity seen with sulfoxaflor in *in vitro* and *in vivo* studies. There is a very high background incidence of

Leydig cell tumours in this particular strain of rat that complicates any interpretation of the incidence of this benign neoplasm with sulfoxaflor exposure. The overall incidence of this neoplasm is within that of the historical control data. There was a significant increase in bilateral LCT incidence only at the highest dose tested. The specificity of the response is very weak and not sufficient for classification with Category 1B.

Similarly, classification with Category 2 is also not proposed based on the overall evidence that supports a mechanism of LCT tumour promotion that is not relevant for humans. The only relevant mode of action (MoA) for sulfoxaflor considered to operate in this case was a weak but chronic dopamine agonism/enhancement by way of neuronal nicotinic acetylcholine receptors (AChR) on dopaminergic neurones. This MoA provides a reasonable explanation with some supporting data. Other modes of action with relevance to human toxicology were refuted. A large body of peer reviewed literature documents LCT promotion with the use of dopamine agonists and large reductions in circulating prolactin. In contrast, there are no reports of testicular cancer associated with the use of dopamine agonists in humans. Also, significant evidence in the public literature exists demonstrating that rat and human Leydig cells differ greatly with respect to their compliment of cell surface receptors – specifically prolactin (PrlR) and gonadotropin releasing hormone (GnRHR) receptors. Stimulation of rat Leydig cells through both PrlR and GnRHR are a rat-specific mechanism by which LCT formation can occur. In summary, there is sufficient evidence that the proposed mechanism of LCT promotion is not relevant for humans and that the occurrence of LCTs in the Fischer 344 rat has no human relevance *per se*.

In view of these considerations, the available evidence is deemed to support a mechanism of action that is not relevant for human health (weak, chronic, enhancement of dopamine release though sulfoxaflor agonism of α4β2 or α4α6β2 central nicotinic acetylcholine receptors, and the subsequent inhibition of prolactin release from the pituitary gland, ultimately leading to a dopamine agonism/enhancement LCT MoA in a uniquely susceptible animal model, the Fischer 344 rat). On the weight of the available evidence, high background incidence and publically available literature, classification for Leydig cell carcinogenicity is not proposed.

DSD: (Directive 67/548/EEC)

Similarly, with reference to Directive 67/548/EEC, classification with Categories 1, 2 or 3 are considered inappropriate. DSD criteria stipulate that a distinction between Category 3 and no classification can be made in cases where *'...the only available tumour data are the occurrence of neoplasms at sites and in strains where they are well known to occur spontaneously with a high incidence.'* This is indeed a common finding and seen specifically with the Fisher F344 rat and the occurrence of Leydig cell tumours.

4.10.5.3 Preputial Gland Tumours

CLP:

In accordance with the criteria in the CLP Regulation, classification in Category 1A for carcinogenicity is not justified as there is no evidence of sulfoxaflor having caused cancer in

humans.

The significance of the apparent higher incidence of preputial gland tumours observed only in male rats in the high dose group cannot be determined and thus the evidence for a direct effect of sulfoxaflor on this organ is weak at best. Preputial gland tumours were not seen in the long-term carcinogenicity study performed with mice. There were no effects in the female rat correlate to the preputial gland – the clitoral gland. There were no effects in other sebaceous type glands (perifollicular sebaceous glands of the skin, Zymbal's gland) in male or female F344/DuCrl rats. Classification in Category 1B is not appropriate.

Classification with Category 2 is also not proposed because there is little to no evidence showing that humans have functional homologues to preputial glands. The effect is seen in a single species – the rat – and may operate via a species specific mechanism of little to no relevance in man.

In view of these considerations, it is considered these effects to be not relevant for human health (slightly higher testosterone level stimulates preputial gland proliferation which, over a lifetime, promotes normal spontaneous tumourigenesis in the rat preputial gland). Classification for Preputial Gland carcinogenicity is not proposed.

DSD: (Directive 67/548/EEC)

Similarly, with reference to Directive 67/548/EEC, classification with Categories 1, 2 or 3 are considered inappropriate.

4.10.5 Conclusions on classification and labelling

Relevant mechanistic data was submitted that provided significant support for the non relevance to humans of the proposed rodent-specific adverse effects. Classification with regard to Directive 67/548/EEC and CLP Regulation (EC) No. 1272/2008 is not thought to be warranted based on the weight of evidence of all the studies in sections 4.10.1 to 4.10.3 and detailed in DAR section B.6.5.

4.11 Toxicity for Reproduction

The reproductive performance in rats was evaluated in a 2-generation study. Developmental toxicity was investigated in the rat and rabbit. A battery of mechanistic studies was conducted to investigate the observation of reduced neonatal survival seen in the reproduction study and the specific morphological alterations seen in the developmental study in the rat. A developmental neurotoxicity study is summarised here and in the neurotoxicity section.

Table 21: Summary table of relevant reproductive toxicity studies

Study	Species/ strain	Dose Ppm (mg/kg bw/d)	NOAEL ppm	LOAEL ppm	Target organ/critical effect	Reference / DAR Reference
2-generation probe study	Rat/CD	0, 100, 500, 1000 ppm (0, 8.12-8.30, 39.5-44.1, and 78.2-81.6)	Parental: 100 (8.12) Offspring: 100 (8.12)	500 ppm (39.5) 500 ppm (39.5)	↑liver wt Hepatocellular hypertrophy ↓post natal survival	Rasoulpour *et al.*, 2010 (081030) B.6.6.1/1
2-Generation Reproduction	Rat/ CD	0, 25, 100, 400 ppm	**Repro**: 100 ppm (6.63 mkd) **Parental**: 100 ppm (6.63 mkd) **Offspring**: 100 ppm (6.63 mkd)	**Repro**: 400 ppm (26.4 mkd) **Parental**: 400 ppm (26.4 mkd) **Offspring**: 400 ppm (26.4 mkd)	**Reproduction**: -Decreased neonatal survival (ca. 2-5%) **Parental toxicity**: Increased liver weight in males at 400 ppm with correlating histopathologic changes **Offspring**: Decreased neonatal survival and a slight delay in preputial separation (puberty onset) in F1 males	Rasoulpour *et al.*, 2010b (091023) B.6.6.1/2
Developmental Toxicity Probe	Rat/ CD	0, 500, 1000, 1500, 2000 ppm (35.4, 68.0, 85.7, 94.2)	**Dam**: N/A **Litter**: N/A	**Dam**: 500 ppm **Litter**: N/A	**Dam**: Reduced feed consumption and body weight gain **Litter**: Not examined in this probe study	Rasoulpour *et al.*, 2008 (081023) B6.6.10/1

Developmental Toxicity Definitive	Rat/ CD	0, 25, 150, 1000 ppm 0, 1.95, 11.5, 70.2)	**Dam**: 150 ppm (11.5 mkd) **Litter**: 150 ppm (11.5 mkd)	**Dam**: 1000 ppm (70.2 mkd) **Litter**: 1000 ppm (70.2 mkd)	**Dam**: 1000 ppm – reduced feed consumption and body weight gain; increased liver weight. **Litter**: ↑postimplantation loss, ↓litter size, ↓foetal body weight; foetal abnormalities (forelimb flexure, bent clavicle, hindlimb rotation, convoluted/hydro-ureter).	Rasoulpour *et al*., 2010c (081024) B.6.6.10/2
Developmental Neurotoxicity study	Rat/Crl: CD(SD)	0, 25, 100 or 400 ppm or 1.8, 7.1, and 27.7 mg/kg/day (gestation) and 1.9, 7.6, and 29.8 mg/kg/day (lactation) (dosing for from gestation day 6 through lactation day 21	100 ppm *equiv. to* 7.4 mg/kg bw /day	400 ppm *equiv. to* 28.8 mg/kg bw /day	-Pup viability index reduced ($p<0.01$) at 400 ppm. -Pup body wt 11.8% lower (PND 1) 6.5% lower (PND 4) at 400 ppm. -Delay in the mean age of attainment of surface righting response at 400 ppm (6.3 days) versus controls at (5.3 days) significant ($p<0.001$).	**Beck, M.J., 2010**B.6.7.2
Developmental Toxicity Gavage Probe	Rabbit/ NZW	0, 10, 15, 20, 25 mkd	**Dam**: 10 mkd **Litter**: N/A	**Dam**: 15 mkd **Litter**: N/A	**Dam**: Body weight loss from GD 7-10 and 39% decrease in GD 7-28 body weight gain compared to controls. **Litter**: Not examined in this probe study.	Rasoulpour & Brooks 2008 (081042) B.6.6.11/1

Developmental Toxicity Dietary Probe	Rabbit/ NZW	0, 500, 1000 ppm (21.7, 36.6)	**Dam**: 500 ppm (21.7 mkd) **Litter**: N/A	**Dam**: 1000 ppm (36.6 mkd) **Litter**: N/A	**Dam**: Body weight loss from GD 7-10 and 33% decrease in GD 7-28 body weight gain compared to controls. **Litter**: Not examined in this probe study.	Rasoulpour & Brooks 2009a (081121) B6.6.11/2
Developmental Toxicity Dietary Definitive	Rabbit/ NZW	0, 30, 150, 750 ppm (1.3, 6.6, 31.9)	**Dam**: 150 ppm (6.6 mkd) **Litter**: 750 ppm (31.9 mkd)	**Dam**: 750 ppm (31.9 mkd) **Litter**: >750 ppm	**Dam**: Decreased feed consumption, body weight gain, and faecal output. **Litter**: No treatment-related effects	Rasoulpour & Brooks 2009b (081043) B6.6.11/3

Table 22: Summary table of mechanistic / Mode of Action reproductive toxicity studies

Study	Species /Strain	Dose PPM (mg/kg bw/d)	Target organ/critical effect	Reference/DAR Reference
A Dietary Reproductive Toxicity Cross-Fostering Study	Rat/Crl :CD(SD)	Gp 1: 0/0 Gp 2: 0/1000 Gp 3: 1000/0 Gp 4: 1000/1000 (81-59) mg/kg bw	**Dam**: ↓feed consumption ↓weight gain **Offspring:** Gp 1: no effect Gp 2: no effect Gp 3: ↓survival Gp 4: ↓survival Pre-natal exposure caused 100% mortality by PND4	Rastoulpour, R.J., Zablotny, C.L., 2010d B.6.6.12.1
A Study of the Effect of XDE-208 on Neonatal Survival in New Zealand White Rabbits	NZW rabbit	0, 750 ppm (29 mg/kg bw)	**Dam:** ↓food consumption/weight gain **Offspring:** No effect	Kuhl, A.J., August, 2009 B.6.6.12.2

Characterization of the agonist effects of XDE-208 on mammalian muscle nicotinic acetylcholine receptors.	*In vitro*	0-3 mM	Rat foetal nAChr binding and agonism. Rat adult/Human adult and foetal nAChr binding and non-agonism	Millar, N., 2010 B.6.6.12.3
Investigation of the critical window of exposure for fetal abnormalities and neonatal survival effects in Crl:CD(SD) rats: Phase 1	Crl:CD (SD) rats	0, 1000 ppm (38.6-76.5 mg/kg)	Exposure from GD 6-16 had no adverse effect. Exposure from GD 16-birth pup death and skeletal defects. Abnormalities reversed in survivors by PND4.	Rasoulpour, R.J., and Zablotny, C. June 2010 B.6.6.12.4
Investigation of the critical window of exposure for fetal abnormalities and neonatal survival effects in Crl:CD(SD) rats: Phase 2	Crl:CD (SD) rats	0, 1000 ppm (63.9-35.7 mg/kg)	Exposure from GD 16-18 and from GD 18-20 had no adverse effect. Exposure from GD 20-birth-pup death and skeletal defects. Abnormalities reversed in survivors by PND4.	Rasoulpour, R.J., and Zablotny, C. June 2010 B.6.6.12.5
Observations on the effects of XDE-208 on the phrenic nerve-hemidiaphragm preparation from new-born rat.	Crl:CD (SD) rats	0, 0.1, 1.0 mM	Sustained contracture of the isolated rat neonate diaphragm	Gibb, A.j., 2010 B.6.6.12.6
Histopathological Evaluation Of Fetal Lung Samples From The Developmental Toxicity Study In Crl:Cd(Sd) Rats.	Crl:Cd(Sd) Rats	0, 1000 ppm	Foetal rat lung normal (rat dev tox study)	Thomas, J. and Marshall, B.S, 2010. B.6.12.7

4.11.1 Effects on fertility

4.11.1.1 Non-human information

Study 1: Rat Multigeneration Study (DAR B.6.6.1/1)

Summary of the dietary probe study:

Groups of 12 male and 12 female Crl:CD(SD) rats were fed diets containing 0, 100, 500, or 1000 ppm sulfoxaflor. These dose levels corresponded to time-weighted average doses for males of 0, 8.26, 40.7 or 79.1 mg/kg/day. The time weighted average doses for females during the various study phases ranged from 0, 8.12-8.30, 39.5-44.1, and 78.2-81.6 mg/kg/day, respectively

Males in the 1000 and 500 ppm dose groups had a treatment-related decrease in feed consumption (approx 10%) and marginal effect on body weight (approx 5%) (1000 ppm only) during the first week of treatment. Females in the 1000 and 500 ppm dose groups had slight treatment-related decreases in body weight gain during the first week of gestation, and females in the 1000 ppm dose group had slightly lower feed consumption during the pre-breeding and gestation phases. Effects in females were slight and not considered adverse.

There was evidence of a slight effect on the liver in males and females. Males of the 1000 and 500 ppm dose groups had increased absolute and relative liver weights that were dose and treatment related (liver weights for females at 1000 ppm were not recorded due to an effect on litter survival; there was no effect on female liver weights at 500 ppm). Treatment-related histological effects were observed in the livers of males given 1000 and 500 ppm and females given 1000 ppm and consisted of a dose-related increase in the severity of hepatocellular hypertrophy, with altered tinctorial properties, involving the centrilobular to midzonal regions of the hepatic lobule. Histological effects were very slight or slight severity in males and very slight in females. In addition, there was treatment-related multifocal hepatocyte vacuolisation (slight severity) in 1000 ppm females.

There were no reproductive or developmental toxicity effects observed in any group up to PND 0 (birth). Shortly after birth, there was a significant decrease in pup survival in the 1000 ppm dose group such that PND 1 survival was 46.3%, compared to 98.3% in controls. In addition, PND 1 pup body weights were significantly decreased (22-25%) relative to controls. The lower pup body weights could be a combination of decreased pup suckling due to foetal toxicity and lower birth weight, which was observed during a developmental toxicity study at the same dose level. By PND 4, eleven of twelve dams had total litter loss resulting in 7.3% pup survival, compared to 95.4% in controls. Because only one of twelve litters remained, this dose group was terminated on PND 6. Pup survival was also affected in the 500 ppm group with 4 of 12 dams losing approximately half of their litters by PND 4, which resulted in a pup survival rate of 81.2% compared to 95.4% in controls. There were no effects on pup body weight in the 500 ppm group. There were no treatment-related effects on any other reproductive parameters at 1000 or 500 ppm, and no reproductive effects at 100 ppm.

Report:	XDE-208: Dietary Reproduction/Developmental Toxicity Screening Test in CRL:CD(SD) Rats.
Author:	R. J. Rasoulpour, A. K. Andrus, C. L. Zablotny, and B. L. Yano.
Date of Report:	28 January 2010
Report Identity:	Study ID: 081030
Testing Facility:	Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan, 48674.
GLP	Yes

Test Substance: XDE-208 (95.6% (wt/wt))

Batch: E2162-34, TSN003725-0001

Guidelines: OPPTS 870.3550, OECD 421

Deviations: None

Acceptable: Yes

Materials and Methods:

Groups of 12 male and 12 female Crl:CD(SD) rats were fed diets containing 0, 100, 500, or 1000 ppm sulfoxaflor. These dose levels corresponded to time-weighted average doses for males of 0, 8.26, 40.7 or 79.1 mg/kg/day. The time weighted average doses for females during the various study phases ranged from 0, 8.12-8.30, 39.5-44.1, and 78.2-81.6 mg/kg/day, respectively. Males were fed the test diets for two weeks prior to breeding and continuing throughout breeding until termination. The females were fed the test diets for two weeks prior to breeding, continuing through breeding (up to two weeks), gestation, lactation and weaning; pups were weaned on postnatal day 21. Effects on gonadal function, mating behavior, conception, development of the conceptus, parturition and postnatal growth and survival were evaluated. In addition, a gross necropsy and histopathologic examination of the adults were conducted with an emphasis on organs of the reproductive system. In the offspring, litter size, pup survival, sex, body weight and the presence of gross external morphological alterations were assessed.

Results:

The test material was homogeneously distributed in feed (SD range 1.4-1.1%). Sulfoxaflor was previously reported to be stable in rodent diets for at least 65 days at concentrations ranging from 0.0005 to 10%. Test diets for the current study were prepared and used within these stability limits.

Analyses of all diets from the initial mix revealed mean concentrations ranging from 96.5 to 101.5% of targeted concentrations.

Parental animals

In-life observations:

Mortality/clinical signs: All animals survived until termination. There were no significant observations made during the cage-side observations (data in study file). There were no treatment-related clinical observations at any dose level during the pre-mating, gestation, or lactation period. Sporadic occurrences of hairloss, mechanical injury, and pale mucous membranes occurred typically in one animal or fewer per dose group.

Body weights/food consumption/test intake: Males in the 1000 ppm group had a treatment-related 5-6% decrease in body weight, relative to controls, from test day 7-28 that was statistically significant on day 7 and 14 and was consistent with decreased feed consumption in this group. There were no treatment-related or statistical significant differences in body weights for males of the 500 or 100 ppm dose groups when compared to controls.

There were no treatment-related effects on the body weight of females of any dose group during the pre-breeding phase; however, during the gestation phase, there was a treatment-related decrease in body weight gain of the 1000 and 500 ppm females (25 and 18%, respectively) when compared to controls, which was statistically significant on GD 0-7. Gestation body weight gain of animals in the 100 ppm group was similar to controls.

Due to complete litter losses in 11 of 12 dams in the 1000 ppm group, body weight and body weight gain measurements during lactation were limited to one dam and could not be evaluated. There were no treatment-related effects on the body weight or body weight gain of females of the 500 or 100 ppm dose groups during the lactation phase. In the 500 ppm group there was a statistically significant increase in mean body weight gain in the LD 4-7 interval, which could be attributed to variability as well as fewer pups/litter and was considered unrelated to treatment because mean body weight gain from LD 1-21 was similar to controls.

There was a treatment-related decrease in feed consumption in males of the 1000 and 500 ppm groups, which was statistically significant on days 1-7. There were no differences in the feed consumption in males of the 100 ppm group when compared to controls. Similar to the males, females in the 1000 ppm group had a treatment-related decrease in feed consumption during the pre-breeding period when compared to controls. Relative to controls, feed consumption differences for females of the 500 and 100 ppm dose group were deemed unrelated to treatment due to lack of a dose-response relationship and because statistical significance was only reached in the 100 ppm group on days 7-14.

During gestation, there was a treatment-related decrease in feed consumption in the 1000 ppm group, which was statistically significant on GD 0-7. Lactation feed consumption in the 1000 ppm group was limited to data from a single dam on LD 1-4 due to litter loss in the days following parturition. There were no treatment-related effects on feed consumption during gestation or lactation for females of the 500 or 100 ppm dose groups.

Males and females were given diets containing 0, 100, 500, or 1000 ppm sulfoxaflor which values corresponded to time-weighted average doses shown below.

Generation	**Males**[a]			**Females**[b]		
	100	**500**	**1000**	**100**	**500**	**1000**
P1	8.26 ±0.80	40.7±2.58	79.1±1.90	8.30	42.2	79.1

a Test substance intake for entire dosing interval
b Test substance intake for premating interval

Table 4.11.1.1.Study 1.1 (DAR Table B.6.6.1.1-1) Reproductive indices and pup survival

	DOSE PPM 0	100	500	1000
NUMBER OF MALES	12	12	12	12
NUMBER OF FEMALES	**12**	**12**	**12**	**12**
MALE MATING INDEX %A	100.0 (12/12)	100.0 (12/12)	100.0 (12/12)	100.0 (12/12)
FEMALE MATING INDEX %B	100.0 (12/12)	100.0 (12/12)	100.0 (12/12)	100.0 (12/12)
MALE CONCEPTION INDEX %C	100.0 (12/12)	100.0 (12/12)	100.0 (12/12)	100.0 (12/12)
FEMALE CONCEPTION INDEX %D	100.0 (12/12)	100.0 (12/12)	100.0 (11/11)	100.0 (12/12)
MALE FERTILITY INDEX %E	100.0 (12/12)	100.0 (12/12)	100.0 (12/12)	100.0 (12/12)
FEMALE FERTILITY INDEX %F	100.0 (12/12)	100.0 (12/12)	100.0 (12/12)	100.0 (12/12)
GESTATION INDEX %G	100.0 (12/12)	100.0 (12/12)	100.0 (12/12)	100.0 (12/12)
GESTATION SURVIVAL INDEX %H	98.3 (173/176)	100.0 (179/179)	99.4 (165/166)	99.4 (177/178)
DAY 1 SURVIVAL INDEX %I	98.3 (173/176)	100.0 (179/179)	94.5 (156/165)	46.3* (82/177)
DAY 4 SURVIVAL INDEX %I	95.4 (165/173)	97.8 (175/179)	81.2 (134/165)	7.3* (13/177)
DAY 7 SURVIVAL INDEX %I (AFTER CULLING)	100.0 (96/96)	97.9 (94/96)	100.0 (93/93)	N/A (0/0)
DAY 14 SURVIVAL INDEX %I (AFTER CULLING)	100.0 (96/96)	97.9 (94/96)	100.0 (93/93)	N/A (0/0)
DAY 21 SURVIVAL INDEX %I (AFTER CULLING)	100.0 (96/96)	97.9 (94/96)	100.0 (93/93)	N/A (0/0)
POSTIMPLANTATION LOSS %J	6.82 ± 9.64	7.38 ± 8.77	6.31 ± 6.32	N/A
SEX RATIO ON DAY 1 MALE:FEMALE	50:50	51:49	48:52	55:45

GESTATION LENGTH (DAYS)	21.4 ± 0.5	21.3 ± 0.5	21.8 ± 0.8	21.7 ± 0.5
TIME TO MATING (DAYS)	2.5 ± 1.1	2.8 ± 1.6	3.4 ± 1.6	3.3 ± 3.6

A (# MALES WHICH MATED RESULTING IN A SPERM + VAGINAL LAVAGE OR PREGNANT/TOTAL # MALES AND FEMALES COHOUSED) X 100%.

B (# FEMALES WHICH WITH A SPERM + VAGINAL LAVAGE OR PREGNANT WITHOUT ADDITIONAL EVIDENCE OF MATING/#FEMALES COHOUSED WITH MALES) X 100%.

C (# MALES SIRED A LITTER/# MALES MATED) X 100%.

d (# FEMALES WITH EVIDENCE OF PREGNANCY/# FEMALES MATED) X 100%.

E (# MALES WHICH SIRED A LITTER/# MALES AND # FEMALES COHOUSED) X 100%.

F (# FEMALES WITH EVIDENCE OF PREGNANCY/# FEMALES COHOUSED WITH MALES) X 100%.

G (# FEMALES DELIVERING A LIVE LITTER/# FEMALES DELIVERING A LITTER) X 100%.

H PERCENTAGE OF NEWBORN PUPS THAT WERE ALIVE AT BIRTH

I (# OF LIVE PUPS ON DAY 1 OR 4/# OF LIVE PUPS ON DAY 0) X 100%.

J mean percent/litter (calculated as [no. implants – no. viable offspring]/no implants) x 100

N/A = NOT APPLICABLE – ANIMALS IN THE HIGH DOSE GROUP WERE SACRIFICED BEFORE SCHEDULED NECROPSY

* STATISTICALLY SIGNIFICANT BASED ON THE CENSORED WILCOXON TEST, ALPHA = 0.0

Reproductive function: There were no treatment-related effects at any dose level on reproductive indices, time to mating, gestation length, or postimplantation loss (Table B.6.6.1-1).

Pathology

Organ weights: Treatment-related organ weight changes were limited to increases in mean absolute and relative liver weights in males given 500 or 1000 ppm. Only mean relative weights were statistically significant (Table B.6.6.1.1-2). Males of the 500 and 1000 ppm dose group had 10% and 14% increases in absolute liver weights and 13% and 19% increases in relative liver weights, respectively, when compared to corresponding control values. The increases in absolute and relative liver weights corresponded with the histologic observation of hepatocyte hypertrophy and therefore, were considered treatment related, but marginally toxicologically relevant. Organ weights for females in the 1000 ppm group were not statistically analysed due to early termination of the group. There were no treatment-related changes in organ weights of females in the 500 ppm dose group or males and females in the 100 ppm dose group.

Table 4.11.1.1. Study 1.2 (DAR Table B.6.6.1.1-2) Liver weights

Sex	**Males**			
Dose (ppm)	0	100	500	1000
Liver, absolute (g)	12.795	12.659	**14.075**	**14.552**
Liver, relative (g/100)	3.144	3.141	**3.544** *	**3.751** *
Sex	Females			
Dose (ppm)	0	100	500	1000 ^
Liver, absolute (g)	12.336	12.183	12.427	12.737
Liver, relative (g/100)	4.132	4.180	4.331	4.473

* Statistically different from control mean by Dunnett's test, alpha = 0.05.
^ No statistical comparisons made due to the early termination of this group.
Bold type indicates the effects judged to be treatment related.

Necropsy: There were no treatment related gross pathologic observations of males and females at any dose level.

Microscopic: Males given 500 or 1000 ppm and females given 1000 pm had marginal treatment-related liver effects. Hepatocellular hypertrophy, with altered tinctorial properties, occurred in the centrilobular/midzonal region of the hepatic lobule of males given 500 or 1000 ppm and females given 1000 ppm. This effect involved almost all animals, was dose related and was very slight or slight in degree in males and very slight in degree in females. In addition, two females given 1000 ppm had a slight degree of hepatocellular vacuolization, compared to the very slight severity that occurred in females given 0, 100, or 500 ppm.

Offspring effects

In-life observations

Clinical signs: Treatment-related litter observations were limited to offspring of the 1000 and 500 ppm dose groups, and were associated with the decreased pup survival at these dose levels. These observations included pups found dead, pale or bluish skin, autolysed pups, cannibalized pups, decreased activity and/or cold to the touch. There were a few other observations occurred at low frequency and bore no relationship to treatment.

Litter size: There were no treatment-related differences in the number of pups born alive or dead in any dose group when compared to control. On PND 1 and 4 there was a treatment-related,

statistically significant, decrease in litter size of the 1000 ppm dose group when compared to controls, which was associated with the pup death (Table B.6.6.1.1-3). There was a slight decrease in mean litter size of the 500 ppm dose group prior to culling on PND 4 (not statistically significant) but considered treatment related due to partial litter loss in 4/12 of the dams at this dose. These decreases in litter size at 500 and 1000 ppm were outside the laboratory's historical control range (PND 1: 12.4 – 15.5 and PND 2: 12.4 – 15.5, n = 17). There were no treatment-related effects on litter size of the 100 ppm dose group when compared to control.

Table 4.11.1.1.Study 1.3 (DAR Table B.6.6.1.1-3) Litter size

DOSE		BORN	BORN						
PPM		LIVE	DEAD	1(BC)	4(BC)	4(AC)	7(AC)	14(AC)	21(AC)
0	MEAN	14.4	0.3	14.2	13.8	8.0	8.0	8.0	8.0
	S.D.	2.9	0.6	3.1	3.0	0.0	0.0	0.0	0.0
	N=	12	12	12	12	12	12	12	12
100	MEAN	14.9	0.0	14.9	14.6	8.0	7.8	7.8	7.8
	S.D.	2.2	0.0	2.2	1.8	0.0	0.4	0.4	0.4
	N=	12	12	12	12	12	12	12	12
500	MEAN	13.8	0.1	13.0	11.2	7.8	7.8	7.8	7.8
	S.D.	1.4	0.3	1.8	3.1	0.9	0.9	0.9	0.9
	N=	12	12	12	12	12	12	12	12
1000	MEAN	14.8	0.1	6.8*	1.6*	1.0*	===	===	===
	S.D.	2.0	0.3	4.6	4.6	2.8	===	===	===
	N=	12	12	12	8	8	0	0	0

* STATISTICALLY DIFFERENT FROM CONTROL MEAN BY WILCOXON'S TEST, ALPHA=0.05.
=== NO DATA AVAILABLE FOR MEAN AND S.D. DUE TO DOSE LEVEL REMOVAL FROM STUDY
(BC) BEFORE CULLING (AC) AFTER CULLING
REDUCED N DUE TO LOSS OF LITTERS

Pup survival and sex ratio: There were no treatment-related effects at any dose level on gestation survival or sex ratio. There was a treatment-related decrease in PND 1 and 4 pup survival of litters from the 1000 and 500 ppm dose groups (Tables B.6.6.1.1-1 and B.6.6.1.1-4).

On PND 1 in the 1000 ppm group, there was one total and ten partial litter losses out of twelve litters resulting in a pup survival rate of 46.3% compared to 98.3% for the control group. By PND 4, the ten dams with partial litter loss had lost the remainder of their litter resulting in eleven total litter losses from the 1000 ppm group (pup survival rate of 7.3% compared to 95.4% for the control group). The single surviving litter was used for pup blood collection on PND 4 and 6 and the dam was used for blood and terminal milk collection on PND 6; therefore, no additional pup survival data were collected on this litter after PND 4.

Table 4.11.1.1.Study 1.4 (DAR Table B.6.6.1.1-4) Pup survival (%)

	0 ppm	100 ppm	500 ppm	1000 ppm
PND 1	98.3	100	**94.5**	**46.3***
PND 4	95.4	97.8	**81.2**	**7.3***
PND 7 [A]	100	97.9	100	N/A
PND 14 [A]	100	97.9	100	N/A
PND 21 [A]	100	97.9	100	N/A

A: After culling
* Statistically different from control by the censored Wilcoxon test, alpha = 0.05.
Bolded value interpreted to be treatment-related.
N/A = Not applicable

Pup survival of the 500 ppm group was affected in a dose-dependent manner. On PND 1, one dam lost 5 of 15 pups and two other dams lost one or two pups, which resulted in a survival rate of 94.5% for the 500 ppm group, compared to 98.3% for the control group. By PND 4, there were four dams that lost approximately half of their litter and two dams that lost one or two pups, which resulted in a PND 4 survival rate of 81.2% for the 500 ppm group, compared to 95.4% for controls. While pup survival rate in the 500 ppm group was not statistically significantly different on PND 1 or 4, it was below historical control values and was deemed treatment-related (PND 1: 96.2 – 100, PND 4: 94.0 – 100, n = 17).

There were no additional treatment-related effects seen in pup survival of litters from the 500 ppm dose group on PND 7, 14, or 21 following culling on PND 4. There were no treatment-related effects seen in pup survival of litters from the 100 ppm dose group on any PND when compared to controls.

Body weight: There was a treatment-related 22-25% decrease in PND 1 male and female pup body weight of the 1000 ppm litters, relative to controls (Tables B.6.6.1.1-4). There were no treatment-related effects on offspring body weight in the 500 and 100 ppm dose groups relative to controls.

Table 4.11.1.1.Study 1.5 (DAR Table B.6.6.1.1-5) Selected pup body weight (g)

	0 ppm	100	500 ppm	1000
PND 1 Males	6.8	6.6	6.7	**5.1***
Percent from Control	NA	-3%	-1%	**-25%**
PND 1 Females	6.3	6.4	6.3	**4.9***
Percent from Control	NA	+2%	0%	**-22%**

***** Statistically Different from Control by Dunnett's Test, Alpha = 0.05.
Bolded value interpreted to be treatment-related.

Offspring postmortem results

Toxicokinetics: There was no sulfoxaflor found in plasma obtained from pups of the control group. There was a dose proportionate increase in the concentration of sulfoxaflor in the plasma of pups from the 100, 500, and 1000 ppm groups. Individual (n = 3) concentrations of sulfoxaflor were equivalent in dam plasma and milk from dams of the 1000 ppm dose group.

Conclusion

Males in the 1000 and 500 ppm dose groups had a treatment-related decrease in feed consumption (approx 10%) and marginal effect on body weight (approx 5%) (1000 ppm only) during the first week of treatment. Females in the 1000 and 500 ppm dose groups had a slight treatment-related decreases in body weight gain during the first week of gestation, and females in the 1000 ppm dose group had slightly lower feed consumption during the pre-breeding and gestation phases. Effects in females were slight and not considered adverse.

There was evidence of a slight effect on the liver in males and females. Males of the 1000 and 500 ppm dose groups had increased absolute and relative liver weights that were dose and treatment related (liver weights for females at 1000 ppm were not recorded due to an effect on litter survival; there was no effect at 500 ppm). Treatment-related histological effects were observed in the livers of males given 1000 and 500 ppm and females given 1000 ppm and consisted of a dose-related increase in the severity of hepatocellular hypertrophy, with altered tinctorial properties, involving the centrilobular to midzonal regions of the hepatic lobule. Histological effects were very slight or slight severity in males and very slight in females. In addition, there was treatment-related multifocal hepatocyte vacuolization (slight severity) in 1000 ppm females.

There were no reproductive or developmental toxicity effects observed in any group up to PND 0 (birth). Shortly after birth, there was a significant decrease in pup survival in the 1000 ppm dose group such that PND 1 survival was 46.3%, compared to 98.3% in controls. In addition, PND 1 pup body weights were significantly decreased (22-25%) relative to controls. By PND 4, eleven of twelve dams had total litter loss resulting in 7.3% pup survival, compared to 95.4% in controls. Because only one of twelve litters remained, this dose group was terminated on PND 6. Pup survival was also affected in the 500 ppm group with 4 of 12 dams losing approximately half of their litters by PND 4, which resulted in a pup survival rate of 81.2% compared to 95.4% in controls. There were no effects on pup body weight in the 500 ppm group. There were no treatment-related effects on any other reproductive parameters at 1000 or 500 ppm, and no reproductive effects at 100 ppm.

Based on these results, the no-observed-effect level (NOEL) for general and reproductive toxicity was 100 ppm

Study 2: Rat multigeneration study (DAR B.6.6.1.2)

Summary: Main study:

Following a dietary probe study, a 2-generation dietary reproduction toxicity study was conducted to evaluate the potential effects of sulfoxaflor on male and female reproductive function, as well as the survival, growth and development of the offspring. Groups of 27/sex Crl:CD(SD) rats were fed diets supplying 0, 25, 100, or 400 ppm sulfoxaflor for approximately ten weeks prior to breeding, and continuing through breeding, gestation and lactation for two generations. Minimal parental toxicity was seen at 400 ppm and consisted of increased absolute and relative liver weights in the P_1 (12.8 and 10.9%, respectively) and P_2 (6.5 and 7.8%, respectively) males. This effect on liver weight correlated with histopathologic findings of very slight to slight centrilobular hepatocyte hypertrophy, often with a very slight increase in individual cell necrosis of centrilobular hepatocytes. No other systemic effects were noted at 400 ppm, and there were no treatment-related effects on P_1 or P_2 parameters in male or female rats at 25 or 100 ppm.

Reproductive effects were limited to 400 ppm and comprised slightly decreased neonatal survival in both generations; this in turn led to a lower percentage of live pups up to culling on PND 4. In addition, there was an apparent treatment-related delay in preputial separation (PPS) for 400 ppm F_1 males. This external marker of male puberty onset is androgen dependent, but the underlying

reason for how sulfoxaflor induced this finding is not known; however, there were no other indications of androgenic or anti-androgenic effects. Taken together, the weight of evidence across androgen-sensitive endpoints led to the conclusion that the data do not support any other sulfoxaflor-mediated anti-androgenic effects. There were no effects on puberty onset or any other parameter of reproductive performance or offspring growth and survival at 25 or 100 ppm.

Toxicokinetic data from LD 4 dams and culled PND 4 pups in the second generation show dose-proportional systemic exposure to sulfoxaflor in dams and their offspring. Plasma concentrations of sulfoxaflor in rat pups were, on average, 32% of the levels measured in the dams.

The lowest-observed-adverse-effect-level (LOAEL) for systemic toxicity was 400 ppm based on hepatic toxicity (increased weight, hypertrophy, and necrosis) in the P_1 and P_2 males. The no-observed-adverse-effect-level (NOAEL) was 100 ppm. The LOAEL for reproductive toxicity was 400 ppm based on decreased pup survival (PND 1-4) in the F_1 and F_2 generations. The NOAEL was 100 ppm. The LOAEL for developmental toxicity was 400 ppm (26.4 mg/kg bw) based on liver effects in P_1 and P_2 males, decreased neonatal survival and delayed preputial separation (PPS). The NOAEL was 100 ppm (6.63 mg/kg bw).

Report:	Two Generation Dietary Reproductive Toxicity Study in CRL:CD(SD) Rats
Author:	R. J. Rasoulpour, C. L. Zablotny, J. Thomas, D. L. Rick, and J. W. Crissman (2010b)
Date of Report:	2 July, 2010
Report Identity:	Study ID: 091023
Testing Facility:	Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan, 48674.
GLP	Yes
Test Substance:	XDE-208 (95.6% (wt/wt))
Batch:	E2162-34, TSN003725-0001
Guidelines:	OPPTS 870.3800, OECD 416
Deviations:	None
Acceptable:	Yes

Materials and Methods:
In a two-generation dietary reproduction toxicity study sulfoxaflor was administered to Crl:CD (SD) rats (27/sex/dose group) at concentrations of 0, 25, 100 or 400 ppm in the diet for approximately ten weeks prior to breeding, and continuing through breeding, gestation and lactation for two generations. In-life parameters included clinical observations, feed consumption, body weights, estrous cyclicity, reproductive performance, pup survival, pup body weights, puberty onset and anogenital distance. In addition, post-mortem evaluations included gross pathology and organ weights in weanlings, toxicokinetic analyses, gross pathology, organ weights, oocyte quantitation and sperm count, motility and morphology, and histopathology, in adults.

Findings:
The test material was homogeneously distributed in feed (SD range 1.8-3.2%). Sulfoxaflor was previouslyreported to be stable in rodent diets for at least 65 days at concentrations ranging from 0.0005 to 10%. Test diets for the current study were prepared and used within these stability limits. The overall mean concentrations of sulfoxaflor in the test diets administered to the animals over the entire study period were 99.6, 96.8, and 98.5% of target in males and 96.5, 95.3, and 96.3% of

target in females in the 25, 100 and 400 ppm dose groups, respectively.

In life observations

Test Substance intake: Males and females were given diets containing 0, 25, 100, or 400 ppm sulfoxaflor. These values corresponded to time-weighted average doses shown in table below:

Table 4.11.1.1. Study 2.1 (DAR Table B6.6.1.1-1): Mean (±SD) Test Substance Intake (mg/kg body weight/day)

Generation	Males[a]			Females[b]		
	25	100	400	25	100	400
P1	1.52±0.44	6.07±1.73	24.6±7.00	1.91±0.351	7.82±1.37	30.5±5.27
P2	1.74±0.702	6.86±2.54	28.1±10.4	2.11±0.503	8.39±1.93	34.4±7.6

a Test substance intake for entire dosing interval
b Test substance intake for premating interval

Body weight/food consumption: There were no treatment-related effects on male or female body weight or body weight gain of any treated groups in either generation. Incidental findings included test day 1 and 8 body weights of P2 males and females at 25 and 400 ppm that were lower than controls and reached statistical significance at various intervals. These observations were attributed to the staggered delivery and weaning of litters (i.e., controls and 100 ppm group weaned earlier, therefore slight older and heavier offspring at start of P2 phase) in these dose levels and not considered an effect of treatment.

Body weight gains of females during gestation or lactation were not affected by treatment in either the P1 or P2 generation. There was a statistically identified increase in LD 7-14 body weight gain for treated groups when compared to controls; however, this observation was deemed spurious and unrelated to treatment as there was no dose response and no correlation with feed consumption.

There were no treatment-related effects on feed consumption at any dose level in males or in females during the pre-mating, gestation or lactation periods throughout the P1 or P2 generations.

Reproductive function: There was no evidence of an effect on oestrous cyclicity at any dose level of sulfoxaflor in either P1 or P2 females.

There were no effects of sulfoxaflor on any sperm analysis parameter at any dose level. P2 testicular sperm concentration in the 400 ppm group was higher than controls (statistically identified); however, this observation was deemed spurious and unrelated to treatment as there was no effect on P2 epididymal sperm counts or concentration and no effect on P1 testicular or epididymal sperm concentration.

There were no effects of treatment at any dose level on mating, conception, fertility or gestation indices, time to mating, or gestation length, in the first or second generation.

Table 4.11.1.1.Study 2.2 (DAR Table B6.6.1.2-2.): Summary Results of Reproductive Performance of P1 Generation

Parameter	Dose (ppm)			
	0	25	100	400
Number of Males	27	26	27	27
Number of Females	27	27	27	27

Male Rating Index (%)[A]	92.6 (25/27)	100.0 (26/26)	100.0 (27/27)	100.0 (27/27)
Female Rating Index (%)[B]	92.6 (25/27)	100.0 (27/27)	100.0 (27/27)	100.0 (27/27)
Male Conception Index (%)[C]	100.0 (25/25)	100.0 (26/26)	88.9 (24/27)	100.0 (27/27)
Female Conception Index (%)[D]	100.0 (25/25)	96.3 (26/27)	88.9 (24/27)	100.0 (27/27)
Male Fertility Index (%)[E]	92.6 (25/27)	100.0 (26/26)	88.9 (24/27)	100.0 (27/27)
Female Fertility Index (%)[F]	92.6 (25/25)	96.3 (26/27)	88.9 (24/27)	100.0 (27/27)
Gestation Index (%)[G]	100.0 (27/27)	100.0 (26/26)	100.0 (24/24)	100.0 (27/27)
Gestation Length (Day)	21.7±0.5	21.5±0.5	21.6±0.5	21.6±0.6
Time to Mating (Day)	2.7±1.3	3.3±2.4	2.3±1.1	3.3±1.8

A (# males with evidence of mating/total # males co-housed with females) X 100%
B (# females with evidence of mating/ # females co-housed with males) X 100%
C (# males with sired a litter/# males mated) X 100
D (# females with evidence of pregnancy/# females mated) X 100
E (# males which sired a litter/# males co-housed with females) X 100
F (# females with evidence of pregnancy/# females co-housed with males) X 100
G (# females which delivered a live litter/# females with evidence of pregnancy) X 100
Data were extracted from Table 57 pages 176 and 177 of the study report

Table 4.11.1.1.Study 2.3 (DAR Table B6.6.1.2-3.): Summary Results of Reproductive Performance of P2 Generation

	Dose (ppm)			
Parameter	**0**	**25**	**100**	**400**
Number of Males	27	25[†]	27	26
Number of Females	27	26	27	27
Male Rating Index (%)[A]	96.3 (26/27)	92.0 (23/25)	100.0 (27/27)	96.2 (25/26)
Female Rating Index (%)[B]	96.3 (26/27)	92.3 (24/26)	100.0 (27/27)	96.3 (26/27)
Male Conception Index (%)[C]	96.2	100.0	92.3	96.0
	(25/26)	(23/23)	(24/26)[^]	(24/25)
Female Conception Index (%)[D]	96.2 (25/26)	100.0 (24/24)	92.3 (24/26)[^]	96.2 (25/26)
Male Fertility Index (%)[E]	92.6 (25/27)	92.0 (23/25)	92.3 (24/26)[^]	92.3 (24/26)
Female Fertility Index (%)[F]	92.6 (25/27)	96.3 (24/26)	92.3 (24/26)[^]	92.6 (25/27)
Gestation Index (%)[G]	100.0 (25/25)	100.0 (24/24)	100.0 (24/24)	100.0 (25/25)
Gestation Length (Day)	3.0±2.4	2.7±1.5	2.3±1.3	2.6±1.0
Time to Mating (Day)	21.6±0.5	21.7±0.5	21.7±0.5	21.5±0.5

A (# males with evidence of mating/total # males co-housed with females) X 100%

B (# females with evidence of mating/ # females co-housed with males) X 100%
C (# males with sired a litter/# males mated) X 100
D (# females with evidence of pregnancy/# females mated) X 100
E (# males which sired a litter/# males co-housed with females) X 100
F (# females with evidence of pregnancy/# females co-housed with males) X 100
G (# females which delivered a live litter/# females with evidence of pregnancy) X 100
^ The # mated was reduced since the pregnancy status for animal 3867 could not be determined due to the proximity of mating to death
† One available male was inadvertently not paired reducing the count from 26 to 25 paired males
Data were extracted from pages Table 58 on pages 178 and 179 of the study report

Toxicokinetics

Data for the 25, 100, and 400 ppm groups were as follows:

- maternal LD 1-4 mean test material intakes were 2.1, 8.5 and 29.2 mg/kg/day, respectively
- maternal LD 4 mean plasma concentrations were 1.1, 4.5 and 15.9 µg sulfoxaflor/g plasma, respectively
- male pup PND 4 mean plasma concentrations were <LLQ (0.6 µg/g), 1.4 and 5.3 µg sulfoxaflor/g plasma, respectively
- female pup PND 4 mean plasma concentrations were <LLQ, 1.5 and 5.8 µg sulfoxaflor/g plasma, respectively
- sulfoxaflor was not detected in any control samples.

Applying the statistical test cited in the statistical analysis section, the quadratic term from the linear regression was not significant when all dose levels (TMI of dams) were included in the regression against sulfoxaflor plasma concentrations of either adult females (dams), male pups, or female pups. Thus there was no deviation from linearity for systemic exposure of the test material to adult female rats or to nursing pups.

These toxicokinetic data show that the systemic exposure to sulfoxaflor based on plasma concentrations was dose-proportional for adult female rats and their offspring. Plasma concentrations of sulfoxaflor in rat pups were ~32% of the levels measured in their respective dams.

Pathology

Organ weights: There were no treatment-related effects on final body weights of males or females at any dose level. Treatment-related organ weight changes were limited to increases in mean absolute and relative liver weights in P1 and P2 males given 400 ppm (Table B6.6.1-4 and Table B6.6.1-5). P1 males in the 400 ppm dose group had 12.8 and 10.9% increases in absolute and relative liver weights, respectively, compared to corresponding controls. The P2 generation was less affected with 6.5 and 7.8% increases in absolute and relative liver weights, respectively, compared to their controls.

P1 males given 400 ppm had a statistically significant increase in absolute, but not relative, kidney weights. This observation was deemed unrelated to treatment and secondary to the higher final body weights in 400 ppm P1 males as there were no treatment-related microscopic effects in the kidney, relative kidney weights were similar, and this finding was not repeated in the kidneys of P2 males. There were no treatment-related changes in organ weights of females in any treatment group or males in the 25 or 100 ppm dose groups for either generation.

Table 4.11.1.1.Study 2.4 (DAR Table B.6.6.1.2-6.): Selected Organ Weight Data (males)

	Dose (ppm)			
	0	25	100	400
Parameter	**P1 Males**			
Final Body Weight (g)	568.0	584.6	578.3	584.4
Absolute Liver (g)	15.090	15.982	15.485	**17.015***
Relative Liver (g/100g bw)	2.654	2.726	2.676	**2.914***
Absolute Kidney (g)	3.895	3.951	3.917	4.182*
Relative Kidney (g/100g bw)	0.688	0.679	0.679	0.716
Parameter	**P2 Males**			
Final Body Weight (g)	568.0	584.6	578.3	584.4
Absolute Liver (g)	15.090	15.982	15.485	**17.015***
Relative Liver (g/100g bw)	2.654	2.726	2.676	**2.914***
Absolute Kidney (g)	3.895	3.951	3.917	4.182*
Relative Kidney (g/100g bw)	0.688	0.679	0.679	0.716

*Statistically Different from Control Mean by Dunnett's Test, Alpha = 0.05.

Bold type indicates the effects are considered treatment-related**.**

Necropsy: There were no gross lesions attributable to administration of the test material in P1 or P2 adults of either sex. All gross pathologic observations were considered spontaneous alterations unassociated with exposure to sulfoxaflor.

Microscopic: The only histopathologic target tissue was the liver, and only males were affected. In P1 and P2 adults, the liver of males administered 400 ppm generally had very slight or slight centrilobular hepatocyte hypertrophy with altered tinctorial properties (Table B6.6.1-7), often accompanied by a very slight increase in individual hepatocyte necrosis in the centrilobular area. The latter finding appeared to be a slight increase in the normal apoptotic cell turnover in the organ. The findings were considered adaptive and non-adverse.

All other histologic findings were considered spontaneous or incidental changes typical of rats of their age, sex, and strain and unassociated with the exposure to sulfoxaflor.

Histologic examination of the reproductive organs of P1 and P2 control and high-dose animals, as well as animals with signs of reduced fertility, did not reveal any treatment-related effects.

Table 4.11.1.1.Study 2.5 (DAR Table B.6.6.1.2-7): Liver Histopathology Finding Incidence, P1 and P2 Males

	Dose (ppm)			
	0	25	100	400
Generation, Tissue, Finding	Males, N = 27			
P1, Liver, centrilobular hepatocyte hypertrophy with altered tinctorial properties	0	0	0	**26**
- Very slight	0	0	0	**2**
-Slight	0	0	0	**24**
P1, Liver, Necrosis, individual hepatocyte, centrilobular multifocal, very slight	9	7	9	**25**
P2, Liver, centrilobular hepatocyte hypertrophy with altered tinctorial properties	0	0	1	**26**
-Very slight	0	0	1	**19**
- Slight	0	0	0	**7**
P2, Liver, Necrosis, individual hepatocyte, centrilobular multifocal, very slight	6	6	4	**12**

Bold type indicates the effects considered to be treatment related.

Offspring data

Viability/clinical signs: Observations made on F1 pups during their respective lactation periods revealed no effects related to treatment. A number of incidental clinical observations bearing no relationship to treatment were observed in the first generation, including one mid-dose F1 pup with a filamentous tail. Due to this single occurrence and lack of dose response, this finding was not considered to be a treatment-related.

There was a treatment-related increase in number of F2 litters with placental tissue attached to dead pups in the 400 ppm group (Table B.6.6.1.8). While this was not observed in the first generation, this finding is deemed consistent with the treatment-related effect of neonatal pup death at this dose level (see Pup Survival section). The remaining observations noted were considered incidental and bore no relationship to treatment.

Table 4.11.1.1.Study 2.6 (DAR Table B.6.6.1.2-8): Selected P2/F2 observation

	Dose Level (mg/kg/day)			
Litter Observations (No. Litters Affected)	0	25	100	400
Within Normal Limits	17	14	17	15
Placental tissue attached	0	0	0	**3**

Bold type indicates the effects considered to be treatment-related

Pup survival and sex ratio: There were no effects of treatment at any dose level on pup sex ratio in the first or second generation. Based on the probe study, a slight decrease in neonatal (PND 1 and 4) survival at 400 was considered treatment-related (Table B.6.6.1.2-9).

While this decrease was not statistically significant, pup survival was clearly affected at 500 and 1000 ppm in the probe study and the values in this study at 400 ppm group were near or slightly below historical control values (95.2% lowest historical control value). There were no additional treatment-related effects seen in pup survival of litters from the 400 ppm dose group after culling on PND 4 or on PND 7, 14, or 21 in either generation.

Table 4.11.1.1.Study 2.7 (DAR Table B.6.6.1.2-9): Pup survival

	F1 Survival			
Dose Level (ppm):	*0*	*25*	*100*	*400*
Gestation Survival (%)	99.2	99.5	99.7	100.0
PND 1 Survival (%)	99.4	99.2	99.1	**98.1**
PND 4 Survival (%)	97.2	97.9	97.1	**95.4**
	F2 Survival			
Gestation Survival (%)	99.7	99.1	98.8	**97.4***
PND 1 Survival (%)	99.7	99.1	98.5	**96.7**
PND 4 Survival (%)	98.8	98.0	97.1	**95.5**
*PND 0-4 Survival (%)^*	98.5	97.1	96.0	**93.0***

* Statistically different from control mean by censored Wilcoxon's test, alpha = 0.05.
^PND 0-4 survival = gestation survival x day 4 survival
Bold type indicates the effects judged to be treatment-related.

As a consequence of this effect on neonatal pup survival, there was a statistically significant decrease in gestation survival index (percentage of live born pups/total pups delivered) in the high-dose F_2 litters. This value was within the historical control range and the finding was not observed in the first generation. Evidence indicating that survival is not affected before birth comes from a cross-fostering study, developmental toxicity study and two critical windows of exposure studies that all demonstrated in utero exposure to sulfoxaflor caused postnatal, and not gestational, death. Given the clear profile of neonatal deaths, effectively coincident with birth and during the very early postnatal period, it is most appropriate to combine the gestation survival index data with PND 1-4 survival to create a combined 'PND 0-4 Survival' category (Table B.6.6.1.2-9) which shows a clear treatment-related, statistically significant decrease in total pup survival at the 400 ppm level. There were no treatment-related effects seen in pup survival of litters from the 100 or 25 ppm dose groups when compared to controls.

There was a slight increase in postimplantation loss in the high-dose second, but not first generation (Table B.6.6.2.1-10). This was not seen in the developmental toxicity study at higher doses and may be related to early neonatal death.

Table 4.11.1.1.Study 2.8 (DAR Table B.6.6.1.2-10) Post-implantation loss

Dose Level (ppm):	0	25	100	400
P1 Postimplantation Loss (%)	5.92±6.75	5.85±7.82	8.23±11.09	7.24±10.6
P2 Postimplantation Loss (%)	7.35±8.3	8.11±8.97	6.87±6.13	**14.03±16.13**

Bold indicates effects considered to be treatment-related, but reflective of early neonatal pup death

Litter size: Due to the increased neonatal death at 400 ppm in both generations, there was a corresponding slight decrease in mean litter size at this dose level in the F1 and F2 litters. Consistent with the neonatal death effect, the number of F2 pups born dead in the high-dose group (400 ppm) was slightly increased (Table B.6.6.1.2-11) but within the historical control range . As mentioned previously, this finding is attributable to early postnatal death known to occur with exposure to sulfoxaflor and not reflective of prenatal death. There were no effects of treatment on the number of pups born live, born dead, or on litter size at any time at 25 or 100 ppm in either generation. Pup body weights were not affected.

Table 4.11.1.1.Study 2.9 (DAR Table B6.6.1.2-11): Mean (±SD) F2 Litter Size

Parameter / Time	Dose Level (ppm) (n=24-25)			
	0	**25**	**100**	**400**
Born Live	13.2±3.1	14.3±2.8	14.3±2.1	**13.4**±3.3
Born Dead	0.0±0.0	0.1±0.3	0.2±0.5	**0.4**±0.9
Day 1 (BC)	13.1±3.1	14.2±2.8	14.1±2.0	**12.9**±3.1
Day 4 (BC)	13.0±3.0	14.0±2.7	13.9±1.8	**12.8**±3.2
Day 7 (AC)	7.9±0.3	8.0±0.2	8.0±0.0	7.8±1.0
Day 14 & 21 (AC)	7.9±0.3	7.9±0.3	8.0±0.0	7.8±1.0

Bold indicates effects considered to be treatment-related, but reflective of early neonatal pup death
BC = Before Culling, AC = After Culling
Data were obtained from Table 60 on page 181 of the study report

Puberty onset: There was an apparent treatment-related, statistically significant, delay (2.4 days) in puberty onset, preputial separation (PPS), for males in the 400 ppm group without a corresponding decrement in body weight. Male puberty onset parameters in the 100 ppm group were nearly identical to control levels. While not statistically significant, PPS in the 25 ppm group occurred 1.8 days later than controls without a corresponding decrement in body weight at attainment. When compared to historical data the day of and body weight at puberty onset for males in both the 400 ppm and 25 ppm groups were outside the historical control range (45.7 days as longest). Due to a lack of dose-response relationship (i.e., no effect at 100 ppm), the findings at 25 ppm were deemed unrelated to treatment. Therefore, there were no treatment-related changes in puberty onset in males at 25 or 100 ppm or in females in any treated group.

Table 4.11.1.1.Study 2.10 (DAR Table B.6.6.1.2-12.) Days to Preputial Separation in P2 Males

Parameter	Dose Level (ppm) (n=25-27)†			
	0	**25**	**100**	**400**
Mean Age (days)	44.6±2.7	46.4±3.4	44.5±2.4	**47.0**±3.4*****
Mean Body Weight (g)	253.6±24.5	265.8±34.5	250.3±26.7	**272.8**±23.9

* Statistically different from control mean by Dunnett's test, alpha = 0.05
† Change in "n" value due to no Data for animals that never separated
Bold type indicates the effects judged to be treatment-related.
Data were obtaine from Table 64 on page 185 of the study report

While an apparent relationship to treatment for the 400 ppm delay in puberty onset cannot be discounted, this finding occurred in isolation. Many factors contribute to puberty onset in male rats; however, a weight of evidence approach across androgen-sensitive endpoints led to the conclusion in this study there was no other indication of a change in androgen status in sulfoxaflor treated rats.

- There were no statistically identified or treatment-related effects on male anogenital distance in F2 males. Anogenital distance is considered one of the most sensitive end points for altered androgen status.
- There was no evidence of hypospadias, ectopic testes, or exposure-related testicular, epididymal, prostate, or seminal vesicle organ weights or histopathologic changes. In addition, there was no effect on qualitative testicular staging.
- There were no significant changes in sperm parameters (spermatid/sperm counts, sperm motility and sperm morphology).

- Sulfoxaflor also had no effects on reproductive indices, including mating, fertility, time to mating, or gestation length.
- There were no test substance-related effects on preputial separation in males in a developmental neurotoxicity study of sulfoxaflor in rats at the same concentration (B.6.7.2.1).
- Sulfoxaflor did not cause an effect in an androgen receptor (AR) transactivation assay conducted at CeeTox Laboratories, Michigan, US.

Thus, there was not a consistent pattern of altered androgenicity in male rats treated with sulfoxaflor. Overall, the data do not support sulfoxaflor-mediated anti-androgenic effects.

Anogenital distance: There were no treatment-related effects on absolute or relative anogenital distance in male or female pups at any dose level.

Offspring post-mortem
There were no treatment-related effects on final body weight or organ weights for males or females in either generation at any dose level. There were no treatment-related gross pathologic observations in any treatment level for either generation. All gross pathologic observations were considered to be spontaneous alterations, unassociated with exposure to sulfoxaflor.

Conclusion
Minimal parental toxicity was seen at 400 ppm and consisted of increased absolute and relative liver weights in the P1 (12.8 and 10.9%, respectively) and P2 (6.5 and 7.8%, respectively) males. This effect on liver weight correlated with histopathologic findings of very slight to slight centrilobular hepatocyte hypertrophy, often with a very slight increase in individual cell necrosis of centrilobular hepatocytes. No other systemic effects were noted at 400 ppm, and there were no treatment-related effects on P1 or P2 parameters in male or female rats at 25 or 100 ppm.

Reproductive effects were limited to 400 ppm and comprised slightly decreased neonatal survival in both generations; this in turn led to a lower percentage of live pups up to culling on PND 4. In addition, there was an apparent treatment-related delay in preputial separation (PPS) for 400 ppm F1 males. This external marker of male puberty onset is androgen dependent, but the underlying reason for how sulfoxaflor induced this finding is not known; however, there were no other indications of androgenic or anti-androgenic effects. Taken together, the weight of evidence across androgen-sensitive endpoints led to the conclusion that the data do not support any other sulfoxaflor-mediated anti-androgenic effects. There were no effects on puberty onset or any other parameter of reproductive performance or offspring growth and survival at 25 or 100 ppm.

Toxicokinetic data from LD 4 dams and culled PND 4 pups in the second generation show dose-proportional systemic exposure to sulfoxaflor in dams and their offspring. Plasma concentrations of sulfoxaflor in rat pups were, on average, 32% of the levels measured in the dams.

The lowest-observed-adverse-effect-level (LOAEL) for systemic toxicity was 400 ppm based on hepatic toxicity (increased weight, hypertrophy, and necrosis) in the P1 and P2 males. The no-observed-adverse-effect-level (NOAEL) was 100 ppm. The LOAEL for reproductive toxicity was 400 ppm based on decreased pup survival (PND 1-4) in the F1 and F2 generations. The NOAEL was 100 ppm. The LOAEL for developmental toxicity was 400 ppm based on liver effects in P1 and P2 males, decreased neonatal survival and delayed preputial separation (PPS). The NOAEL was 100 ppm.

Parental LOAEL = 400 ppm (24.6 mg/kg bw)((slight liver effects)
Fertility LOAEL = 400 ppm preputial separation)
Fertility NOAEL = 100 ppm (6.07 mg/kg bw/day).
Developmental LOAEL = 400 ppm (increased post-implantation loss/decreased post natal survival/placenta attached to dead pups/decreased pup weight.
Developmental NOAEL = 100 ppm.

4.11.1.2 Human information

No data available.

4.11.2 Developmental toxicity

4.11.2.1 Non-human information

Study 1a: Rat developmental study (DAR B.6.6.10/1 and 2)
Following a dietary probe study, potential developmental toxicity of sulfoxaflor was investigated in the rat. Groups of 26 time-mated female Crl:CD(SD) rats were administered diets containing 0, 25, 150, or 1000 ppm sulfoxaflor on gestation day (GD) 6 through 21 corresponding to time-weighted average doses of 0, 1.95, 11.5, or 70.2 mg/kg/day, respectively, in order to evaluate the potential maternal and developmental toxicity of this compound. In-life maternal study parameters included clinical observations, body weight, body weight gain, and feed consumption. On GD 21, all rats were euthanized and each dam and foetus was examined for gross pathologic alterations. In addition, blood was collected from dams and foetuses to determine blood levels of the test material. Liver, kidneys and gravid uterine weights were recorded, along with the number of corpora lutea, uterine implantations, resorptions and live/dead foetuses. All foetuses were weighed, sexed and examined for external alterations. Approximately one half of the foetuses were examined for visceral and craniofacial alterations, while skeletal examinations were conducted on the remaining foetuses.

Administration of 1000 ppm sulfoxaflor in rodent feed resulted in maternal and developmental toxicity. Maternal toxicity was evidenced by decreases in body weight and body weight gains, relative to controls, with concomitant decreased feed consumption, throughout the treatment period, and increased relative liver weights. Developmental toxicity was evidenced by decreases in foetal body weight and gravid uterine weight. In addition, clear increases in several foetal abnormalities (forelimb flexure, bent clavicle, hindlimb rotation, convoluted ureter, and hydroureter) occurred, which have subsequently been shown to reverse by postnatal day four. The terminal plasma concentrations of sulfoxaflor in both dam and foetal blood were dose-proportional throughout the entire range of dietary exposure concentrations with similar levels between the maternal and foetal blood compartments. Administration of 150 or 25 ppm sulfoxaflor in rodent feed produced no treatment-related maternal toxicity and no indications of embryo/foetal toxicity or teratogenicity. Therefore, under the conditions of this study, the no-observed-effect level (NOEL) for maternal and embryo/foetal toxicity was 150 ppm.

The developmental toxicity study in the rat is classified acceptable and satisfies the guideline requirement for a developmental toxicity study (OPPTS 870.3700; OECD 414) in [rats].

Report:	Dietary Developmental Toxicity Probe Study in Crl:CD(SD) Rats.
Author:	R. J. Rasoulpour, Ph.D., V. A. Marshall, B.S. and B. L. Yano, D.V.M., Ph.D.

Date of Report:	22 October 2008
Report Identity:	Study ID: 081023
Testing Facility:	Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan, 48674.
GLP	Yes
Test Substance:	XDE-208 (95.6% (wt/wt); as two diastereomers in 48.4/47.4% ratio.
Batch:	E2162-34, TSN003725-0001
Guidelines:	OPPTS 870.3700, OECD 414
Deviations:	None
Acceptable:	Yes

Materials and Methods:
The purpose of this study was to make a preliminary evaluation of the maternal toxicity and embryo/foetal lethality potential of sulfoxaflor in Crl:CD(SD) rats following dietary administration. Results from this study will be used to set dose levels for a subsequent dietary developmental toxicity study in rats. Groups of seven time-mated female Crl:CD(SD) rats were administered XDE-208 in rodent feed at targeted dose levels of 0, 500, 1000, 1500, or 2000 ppm corresponding to time-weighted average concentrations of 0, 35.4, 68.0, 86.7, and 94.2 mg/kg/day, respectively, on gestation day (GD) 6 through 21. In-life parameters evaluated for all groups included clinical observations, body weight, body weight gain, and feed consumption. On GD 21, all surviving rats were euthanized and examined for gross pathologic alterations. Liver and kidney weights were recorded, along with the number of corpora lutea, implantations, resorptions, and live/dead foetuses.

Results:
Administration of sulfoxaflor to time-mated Crl:CD(SD) rats resulted in excessive system toxicity at 1500 and 2000 ppm evidenced by body weight loss, decreased body weight gain, and decreased feed consumption. Therefore, all animals in these groups were euthanized for humane reasons on GD 13 with no further collection of data. Animals in the 1000 ppm dose group had transient and less severe treatment-related decreases in body weight/body weight gain, decreased feed consumption, and increased relative liver weights. The 500 ppm dose group had treatment-related, transient, decreased feed consumption during the first three days of treatment; however, the body weights and body weight gains remained comparable to controls. There were no treatment-related clinical observations in any group tested and no treatment-related gross pathology observations, effects on pregnancy rates, numbers of corpora lutea, implantations, increase in resorption rate, or litter size and no indication of embryo/foetal lethality in animals given 500 or 1000 ppm sulfoxalfor.

Table 4.11.2.1.Study 1a.1 (DAR Table B.6.6.10.1-1) Maternal Body Weight Gain (g)

		DAYS OF GESTATION							
DOSE									
PPM		0-6	6-9	9-12	12-15	15-18	18-21	6-21	0-21
0	MEAN	43.9	18.0	22.1	17.4	41.4	54.4	153.4	197.3
	S.D.	10.1	7.4	6.7	6.8	4.9	12.1	27.0	30.4
	N=	7	7	7	7	7	7	7	7

500	MEAN	39.2	16.7	19.6	18.0	35.9	45.9	136.1	175.3
	S.D.	5.4	2.7	2.1	2.9	8.9	5.9	15.1	14.1
	N=	6	6	6	6	6	6	6	6
1000	MEAN	41.5	2.5*	22.2	14.7	40.5	47.9	127.7	169.2
	S.D.	6.0	4.3	3.4	4.7	5.5	6.3	11.8	15.8
	N=	7	7	7	7	7	7	7	7
1500	MEAN	37.4	-1.3*	15.4	===	===	===	===	===
	S.D.	5.9	6.3	2.8	===	===	===	===	===
	N=	7	7	7	0	0	0	0	0
2000	MEAN	35.0	-13.8*	12.8	===	===	===	===	===
	S.D.	4.1	7.4	8.5	===	===	===	===	===
	N=	7	7	7	0	0	0	0	0

==========

* Statistically different from control mean (dunnett's test, $\alpha = 0.05$).

= No data available for mean and SD pregnancy status undetermined for the following group(s): 1500, 2000

[a] Data extracted from pgs (31) of the study report

Table 4.11.2.1.Study 1a.2 (DAR Table B6.6.10.1.1-2) Caesarean section observations

DOSE (PPM)	0	500	1000	1500	2000
NUMBER BRED	7	7	7	7	7
% PREGNANT[a]	7/ 7(100.0%)	6/7 (85.7%)	7/7 (100.0%)	N/A	N/A
NUMBER OF DEATHS	0	0	0	0	0
NUMBER MORIBUND	0	0	0	0	0
NUMBER ABORTED	0	0	0	0	0
NUMBER REMOVED EARLY	0	0	0	7	7
PREGNANCIES DETECTED BY STAIN[b]	0/0	0/1	0/0	0/0	0/0
NUMBER OF LITTERS TOTALLY RESORBED	0	0	0	0	0
NUMBER OF LITTERS WITH VIABLE FOETUSES	7	6	7	N/A	N/A
NUMBER OF CORPORA LUTEA/DAM[c]	15.3 ± 2.8	14.7 ± 2.1	14.6 ± 1.5	N/A	N/A
NUMBER OF IMPLANTATIONS/DAM[c]	13.4 ± 1.5	13.8 ± 1.7	13.6 ± 1.3	N/A	N/A
MEAN % PREIMPLANTATION LOSS[d]	10.8 ± 12.5	5.2 ± 7.0	6.2 ± 10.8	N/A	N/A
NUMBER OF RESORPTIONS/LITTER[c,f]	0.1 ± 0.4	0.8 ± 0.8	0.1 ± 0.4	N/A	N/A
RESORPTIONS/LITTERS WITH RESORPTIONS[f]	1.0 (1/1)	1.3 (5/4)	1.0 (1/1)	N/A	N/A
MEAN % POSTIMPLANTATION LOSS[e]	1.0 ± 2.5	5.7 ± 5.3	1.0 ± 2.9	N/A	N/A
VIABLE FOETUSES/LITTER[c]	13.3 ± 1.4	13.0 ± 1.4	13.4 ± 1.4	N/A	N/A

a No. of animals with visible implantations/total no bred
b No. of females detected as being pregnant after sodium sulphide staining/total no. stained
c. No of females detected.
d Mean percent/litter (calculated as [(no. Corpora lurea – no. Implantations)/ no. Corpora lurea] x 100
e mean %/litter (calculated as [no. Implantations - live born pups/no. Implantations] x 100
f Not statistically analyses
N/A Not Applicable due to early termination of dose group
There were no statistically identified differences from control $\alpha = 0.05$
A Data extracted from pg (41) of the study report.

Conclusion:

Administration of sulfoxaflor to time-mated Crl:CD(SD) rats resulted in excessive systemic toxicity at 1500 and 2000 ppm as demonstrated by body weight loss, decreased body weight gain, and decreased feed consumption. Therefore, all animals in these groups were euthanized for humane reasons on GD 13 with no further collection of data. Animals in the 1000 ppm dose group had transient and less severe treatment-related decreases in body weight/body weight gain, decreased feed consumption, and increased relative liver weights. The 500 ppm dose group had treatment-related, transient, decreased feed consumption during the first three days of treatment; however, the body weights and body weight gains remained comparable to controls. There were no treatment-related clinical observations in any group tested and no treatment-related gross pathology observations, effects on pregnancy rates, numbers of corpora lutea, implantations, increase in resorption rate, or litter size and no indication of embryo/foetal lethality in animals given 500 or 1000 ppm sulfoxaflor.

Based on the results of this study dose levels of 25, 150, and 1000ppm sulfoxaflor were selected for the definitive developmental toxicity study.

Study 1b: Rat developmental study (DAR B.6.6.10/1 and 2)

Report:	Dietary Developmental Toxicity Study in CRL:CD(SD) Rats.
Author:	R. J. Rasoulpour, Ph.D., V. A. Marshall, B.S. and S. A. Saghir, M.S.P.H., Ph.D.
Date of Report:	16 June 2010
Report Identity:	Study ID: 081024
Testing Facility:	Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan, 48674.
GLP	Yes
Test Substance:	XDE-208 (95.6% (wt/wt); as two diastereomers in 48.4/47.4% ratio.
Batch:	E2162-34, TSN003725-0001
Guidelines:	OPPTS 870.3700, OECD 414
Deviations:	None
Acceptable:	Yes

Materials and Methods:
In a prenatal developmental study, groups of 26 time-mated female Crl:CD(SD) rats were administered diets containing 0, 25, 150, or 1000 ppm sulfoxaflor on gestation day (GD) 6 through 21 corresponding to time-weighted average doses of 0, 1.95, 11.5, or 70.2 mg/kg/day, respectively, in order to evaluate the potential maternal and developmental toxicity of this compound. In-life maternal study parameters included clinical observations, body weight, body weight gain, and feed consumption. On GD 21, all rats were euthanized and each dam and foetus was examined for gross pathologic alterations. In addition, blood was collected from dams and foetuses to determine blood levels of the test material. Liver, kidneys and gravid uterine weights were recorded, along with the number of corpora lutea, uterine implantations, resorptions and live/dead foetuses. All foetuses were weighed, sexed and examined for external alterations. Approximately one half of the foetuses were examined for visceral and craniofacial alterations, while skeletal examinations were conducted on the remaining foetuses.

Results
Maternal Toxicity
Clinical signs: : Examinations performed on all animals revealed no treatment-related findings. There were some sporadic, transient alterations that were considered incidental and not related to treatment.
Body weight and food consumption: In the 1000 ppm group there was a treatment-related, statistically significant, 9.0% decrease in mean body weights, relative to controls, on GD 21. Mean body weight gains throughout the treatment period (GD 6-21), were decreased 22% relative to controls, which correlated with decreased feed consumption. There were no treatment-related effects on mean body weights or body weight gains in the 25 and 150 ppm groups when compared to controls. There was a treatment-related statistically significant decrease (10-39%) in mean feed consumption in the 1000 ppm group, compared to controls at all intervals, except GD 3-6 (pre-test material administration), GD 15-18 and GD 18-19. There were no treatment-related differences in the amount of feed consumed by animals in the 25 or 150 ppm groups compared to controls.

Table 4.11.2.1.Study 1b.1 (DAR Table B.6.6.10.2-1.): Maternal Body Weight Gain Summary (grams)

Days of Gestation									
Dose PPM		**0-6**	**6-9**	**9-12**	**12-15**	**15-18**	**18-21**	**6-21**	**0-21**
0	Mean S.D. N=	36.6 6.9 24	15.5 6.0 24	23.2 6.1 24	20.5 5.4 24	41.2 5.8 24	52.5 9.3 24	152.9 18.5 24	189.4 22.4 24
25	Mean S.D. N=	37.8 6.2 23	15.7 3.6 23	22.7 6.8 23	20.2 4.4 23	42.1 5.5 23	49.5 12.6 23	150.1 18.2 23	187.8 19.2 23
150	Mean S.D. N=	38.5 6.9 25	17.3 5.1 25	22.3 5.3 25	20.1 6.3 25	41.8 7.2 25	52.5 6.1 25	154.0 17.1 25	192.5 19.5 25
1000	Mean S.D. N=	33.8 10.0 25	0.8$ 8.4 25	19.0 7.6 25	18.3 5.5 25	38.1 8.5 25	42.4$ 5.7 25	118.6* (22%) 15.2 25	152.4* (20%) 18.3 25

* Statistically different from control mean by Dunnett's test, alpha=0.05.
$ Statistically different from control mean by Wilcoxon's test, alpha = 0.05
(%) indicates percent change compared to concurrent control group

Table 4.11.2.1.Study 1b.2 (DAR Table B.6.6.10.2-2): Maternal Body Weights Summary (grams)

Day of Gestation									
DOSE PPM		**0**	**6**	**9**	**12**	**15**	**18**	**21**	**21[a]**
0	Mean S.D. N=	236.0 7.3 24	272.5 11.7 24	288.0 15.2 24	311.2 17.1 24	331.7 21.0 24	372.9 20.8 24	425.4 27.2 24	319.0 23.2 24
25	Mean S.D. N=	232.9 6.4 23	270.6 9.8 23	286.3 10.6 23	309.0 12.9 23	329.1 14.3 23	371.2 15.9 23	420.7 20.4 23	317.9 20.3 23
150	Mean S.D. N=	234.0 6.0 25	272.6 8.3 25	289.9 10.3 25	312.2 11.8 25	332.3 13.6 25	374.1 16.5 25	426.6 20.3 25	319.9 18.7 25
1000	Mean S.D. N=	234.9 7.3 25	268.6 12.0 25	269.4* (↓7%) 11.0 25	288.4* (↓7%) 13.1 25	306.7* (↓7%) 14.5 25	344.8* (↓7%) 17.2 25	387.2* (↓9%) 18.7 25	294.9* (↓7%) 16.4 25

* Statistically different from control mean by Dunnett's test, alpha=0.05.
a = terminal body weight - gravid uterine weight
(%) indicates percent change compared to concurrent control group
Data extracted from pg 37 of the study report

Test material intake: Rats were given 0, 25, 150, or 1000 ppm sufloxaflor in rodent feed, which corresponded to time-weighted average doses of 0, 1.95, 11.5, or 70.2 mg/kg/day.

Toxicokinetics: The increase in plasma concentration of sulfoxaflor was proportional to dose throughout the 25, 150, and 1000 ppm dose groups. Comparison between sulfoxaflor concentration in the maternal and foetal compartments on GD 21 revealed foetal plasma levels which were 76, 82, and 85% of maternal plasma in the low-, mid-, and high-dose groups, respectively.

Pathology: There were no treatment-related gross pathologic observations in any group tested. All observations were deemed spurious and not associated with exposure to sulfoxaflor. In the 1000

ppm group there was a treatment-related, statistically significant, 6.1% increase in relative liver weights, compared to control. There were no statistically identified differences in any of the measured parameters in the 25 and 150 ppm groups.

Caesarean section data: In the 1000 ppm group there were treatment-related statistically significant decreases in foetal weights and gravid uterine weights. The mean number of viable foetuses/litter in the 1000 ppm group (12.3) was slightly lower (statistically significant) than that of the control group (13.3). The statistically identified decrease in viable foetuses/litter is considered unrelated to treatment by the investigator mainly due to a low percentage of postimplantation loss in the control group (1.4% - close to the minimum value among recent historical controls (1.2%). The apparent increase in post-implantation loss was not dose-related and within the range of the historical data provided. There were no treatment-related effects on any reproductive parameters measured in the 25 and 150 ppm groups.

Table 4.11.2.1.Study 1b.3 (DAR Table B.6.6.10.2-3): Caesarean Section Observations

Dose (ppm)	**0**	**25**	**150**	**1000**
Number Bred	26	26	26	26
% Pregnant [a]	24/26 (92.3%)	23/26 (88.5%)	25/26 (96.2%)	25/26 (96.2%)
Number of Deaths	0	0	0	0
Number Moribund	0	0	0	0
Number Aborted	0	0	0	0
Number Removed Early	0	0	0	0
Pregnancies Detected by Stain [b]	0/2	0/3	0/1	0/1
Number of Litters Totally Resorbed	0	0	0	0
Number of Litters with Viable Foetuses	24	23	25	25
Number of Corpora Lutea/Dam [c]	14.1 ± 1.9	14.1 ± 1.7	14.3 ± 1.4	13.5 ± 1.6
Number of Implantations/Dam [c]	13.5 ± 1.8	13.3 ± 1.6	13.9 ± 1.5	13.0 ± 1.5
Mean % Preimplantation Loss [d]	3.6 ± 5.8	5.6 ± 7.0	2.8 ± 4.4	3.4 ± 6.3
Number of Resorptions/Litter [c,f]	0.2 ± 0.4	0.7 ± 1.0	0.6 ± 1.0	0.7 ± 0.9
Resorptions/Litters with Resorptions [f]	1.0 (5/5)	1.7 (15/9)	1.8 (16/9)	1.5 (18/12)
Mean % Postimplantation Loss [e]	1.4 ± 2.8	4.9 ± 7.8	5.1 ± 8.1	5.2 ± 6.4
Viable Foetuses/Litter [c]	13.3 ± 1.7	12.7 ± 1.9	13.3 ± 2.1	12.3 ± 1.3[$]
Foetal Weight – Males (g) [c]	5.94 ± 0.31	6.02 ± 0.31	6.02 ± 0.25	5.29 ± 0.32*
Foetal Weight – Females (g) [c]	5.67 ± 0.26	5.71 ± 0.37	5.63 ± 0.28	4.99 ± 0.27*
Foetal Weight – Sexes Combined (g) [c]	5.79 ± 0.26	5.87 ± 0.34	5.83 ± 0.25	5.12 ± 0.30*
Gravid Uterine Weight (g) [c]	106.38 ± 12.18	102.80 ± 13.48	106.62 ± 15.17	92.34 ± 10.00*
Sex Ratio (M:F)	48:52	52:48	49:51	44:56

[a] No. of Females With Visible Implantations/Total No. Bred.

[b] No. of Females Detected as Being Pregnant After Sodium Sulfide Stain/Total No. Stained.

[c] Mean ± S.D.

[d] Mean Percent/Litter (Calculated As [(No. Copora Lutea - No. Implantations)/No. Corpora Lutea] X 100

[e] Mean Percent/Litter (Calculated As [(No. Implantations – Live Born Pups / No Implantations] X 100

[f] Not Statistically Analysed.

$ Statistically Different from Control Mean by Wilcoxon's Test, Alpha=0.05.

* Statistically Different from Control Mean by Dunnett's Test, Alpha=0.05.

Table 4.11.2.1.Study 1b.4 (DAR Table B.6.6.10.2-4): Caesarean Section Observations Historical Control (gavage studies)

	1 6/2004	2 7/2004	3 6/2005	4 8/2005	5 10/2005	6* 8/2005	7* 2/2009

Mean % Post implantation Loss	3.9 ± 7.5	3.6 ± 4.9	1.2 ± 3.7	7.0 ± 8.3	7.1 ± 9.9	8.2 ± 12.6	5.2 ± 6.9
Viable Foetuses/Litter	12.9 ± 2.4	13.0 ± 1.6	13.0 ± 2.2	12.2 ± 2.1	12.3 ± 2.1	12.6 ± 1.9	11.0 ± 3.7

*Data collected from probe studies.
Data extracted from pg 26 of the study report.

Foetal observations
In the 1000 ppm group there were treatment-related increases in the incidence of foetal alterations (described below). There were no treatment-related effects on the incidence of any foetal alterations in the 25 and 150 ppm groups.

Study authors note:
Foetal Abnormalities: A malformation is defined by the U.S. Environmental Protection Agency's developmental toxicity risk assessment guidance document, as "a permanent structural change that may adversely affect survival, development or function." The protocol for this study cites this statement and adds that a malformation would also have "occurred at a relatively low incidence in the specific species/strain."
The foetal abnormalities of forelimb flexure (>90º flexure), slight forelimb flexure (45-90º flexure), hindlimb rotation, convoluted ureter, hydroureter, and bent clavicle observed in this study do not match this standard definition. They are unusual in that they were shown in subsequent studies to reverse by postnatal day four and therefore are not a "permanent structural change". For this reason and to avoid confusion with abnormalities that are universally recognized to be consistent with the term 'malformation' they have been described as "transient alterations" throughout this report and footnoted as such in appropriate tables.
In addition, it is important to point out that there was an initial problem with the procedure used in the external examinations, specifically as it related to the evaluation of forelimb flexures. This procedural issue was rectified after the first four litters (1 litter/dose group) had been evaluated. Based on our expert judgment together with guidance from independent, internationally recognized third party experts, we have excluded these data were excluded from the summary tables and the data interpretation described below. However, the forelimb flexure (<90º) and hindlimb rotation were severe enough such that these conditions would not have occurred spontaneously, were considered chemical related, and therefore were included in the data analysis. All of the data are presented in the study report with a full account of data that were excluded, a note to the study file, and a letter from the third party experts.

External examination: In the 1000 ppm group, 129/295 foetuses had treatment-related external alterations. Approximately 40% of the foetuses in this group (122/295) had unilateral or bilateral forelimb flexure and twelve foetuses had hindlimb rotation abnormalities. In addition, approximately 60% of the foetuses (154/248) had unilateral or bilateral slight forelimb flexure (variation). The incidences of severe forelimb flexure, hindlimb rotation, and slight forelimb flexure were statistically significant and considered treatment-related. Foetuses in this group exhibited a contracted or hunched posture of the body, limbs, and neck. This did not appear to be a structural defect, but instead, was noted during visceral examination as a difficulty in laying the foetuses flat due to skeletal muscle contracture. Despite the fact that the study team was blinded to dose levels, litters from dams given 1000 ppm sulfoxaflor were easily distinguishable from other dose groups on the basis of this appearance. There were no treatment-related external alterations in the 25 or 150 ppm groups.
There was one foetus in the 150 ppm group with subdermal hematoma that was unrelated to treatment and of no toxicological significance.

Table 4.11.2.1.Study 1b.5 (DAR Table B6.6.10.2-5): Foetal External Examinations

		0 ppm	25 ppm	150 ppm	1000 ppm
Forelimb Flexure^	F	0/320 (0.0)	0/278 (0.0)	0/332 (0.0)	**122/295 (41.4)***
	L	0/24 (0.0)	0/22 (0.0)	0/25 (0.0)	**23/24 (95.8)**
Slight Forelimb Flexure^	F	0/265 (0.0)	0/225 (0.0)	0/283 (0.0)	**154/248[A] (62.1)***
	L	0/20 (0.0)	0/18 (8.3)	0/21 (0.0)	**20/20[A] (100.0)**
Hindlimb Rotation^	F	0/320 (0.0)	0/278 (0.0)	0/332 (0.0)	**12/295 (4.1)***
	L	0/24 (0.0)	0/22 (0.0)	0/25 (0.0)	**7/24 (29.2)**

Bold type indicates treatment-related effects
F = Foetus L = Litter
^ indicates a transient alteration
* Statistically different from control mean by censored Wilcoxon's Test, α= 0.05.
[A] Incidences of slight forelimb flexure from first 4 litters/dose group examined were not included. See evaluators comment below.

Reliability of the study:

1. During the second week of the caesarean sections, a low dose litter (animal 6379) exhibited anomalies previously only observed in high dose litters, and a high dose litter (animal 6431) showed no signs of anomalies. Sulfoxaflor blood levels of these litters and from an additional high and low dose litter, confirmed there was a mix-up and that the diets for 6379 and 6431 had been switched. Data from these litters was not included in the evaluation of this study.
2. On the first day of foetal examinations (October 6, 2008), foetuses from the first 4 litters/dose group were euthanized prior to evaluation (as per the agreed procedure which requires the euthanasia of foetuses as quickly as possible after caesarean section). A large number of foetuses had varying degrees of forelimb flexure (slight forelimb flexure (SFF flexure of 45-90°) and forelimb flexure (FF flexure >90°). Due to the subtle nature of many of these flexures and their high frequency (normally they occur very sporadically), the accuracy and inter-observer consistency for noting these limb observations in dead foetuses was brought into question. After discussions with study personnel and internal experts, which continued into the second of eight days of foetal examinations, the decision was made to evaluate forelimb flexure only in live foetuses for the remaining litters due to concerns about the accuracy of the data when evaluating these anomalies in euthanized foetuses. Based on expert third party guidance, it was decided to exclude all instances of SFF (variation) and to include all instances of FF (malformation) from the litters examined after euthanasia in the data analysis.

Visceral examination: In the 1000 ppm group, 19/149 foetuses examined had unilateral or bilateral convoluted ureter, the incidence of which reached statistical significance and was deemed treatment-related. Two of these foetuses also had hydroureter. There was a single foetus in the 25 ppm group that had bilateral convoluted ureter; however, due to the low incidence (1/139 foetuses) and lack of dose response this was deemed unrelated to treatment. There were no visceral alterations in the control and 150 ppm groups.

Skeletal examination: There were 40/133 foetuses in the 1000 ppm group with unilateral or bilateral bent clavicle, which co-occurred with limb abnormalities in 35/40 foetuses. This finding was statistically significant and considered treatment-related. There was a statistically identified, treatment-related increase in the incidence of one minor skeletal variation, fused sternebrae (6/133 foetuses), in the 1000 ppm group. There was one foetus in the 150 ppm group with fused sternebrae which was considered to be spurious and unrelated to treatment due to the low incidence (1/159 foetuses). There were two foetuses in the 150 ppm group, one in the 25 ppm group, and two in the control group with skeletal malformations. In the 150 ppm group one foetus (6398) had misaligned caudal vertebrae and another foetus (6415) had an extra lumbar vertebra. In the 25 ppm group one

foetus (6378) had a forked 12th rib associated with hemivertebra of the 13th thoracic vertebra and fused thoracic vertebrae. These malformations in the 25 and 150 ppm groups were isolated findings that were not seen at the high dose level and were unrelated to treatment. In the control group one foetus (6352) had misaligned caudal vertebrae and one foetus (6360) had and extra lumbar vertebra. There were no craniofacial alterations in any of the foetuses from any of the dose groups.

Table 4.11.2.1.Study 1b.6 (DAR Table B.6.6.10.2-6) Visceral Examination

		0 ppm	25 ppm	150 ppm	1000 ppm
Convoluted Ureter^	F L	0/168 (0.0) 0/24 (0.0)	1/139 (0.7) 1/22 (4.3)	0/173 (0.0) 0/25 (0.0)	**19/149 (12.8)*** **7/24 (29.2)**
Hydroureter^	F L	0/168 (0.0) 0/24 (0.0)	0/139 (0.0) 0/22 (0.0)	0/173 (0.0) 0/25 (0.0)	[A]**2/149 (1.2)** **2/24 (8.3)**

Bold type indicates treatment-related effects F = Foetus L = Litter
^ indicates a transient alteration
[A] These foetuses also had convoluted ureter
* Statistically different from control mean by censored Wilcoxon's Test, $\alpha = 0.05$.

Table 4.11.2.1.Study 1b.7 (DAR Table B.6.6.10.2-7) Skeletal Examination

		0 ppm	25 ppm	150 ppm	1000 ppm
Bent Clavicle^	F L	0/152 (0.0) 0/24 (0.0)	0/126 (0.0) 0/22 (0.0)	0/159 (0.0) 0/25 (0.0)	**40/133 (30.1)*** **17/24 (70.8)**
Fused Sternebrae	F L	0/152 (0.0) 0/24 (0.0)	0/126 (0.0) 0/22 (0.0)	1/159 (0.6) 1/25 (4.0)	**6/133 (4.5)*** **5/24 (20.8)**

Bold type indicates treatment-related effects F = Foetus L = Litter
^ indicates a transient alteration
* Statistically different from control mean by censored Wilcoxon's Test, $\alpha = 0.05$.

Conclusions

Administration of 1000 ppm sulfoxaflor in rodent feed resulted in some maternal toxicity and developmental toxicity. Some maternal toxicity was seen at the high dose as indicated by decreases in body weight (8%) and body weight gains (22%), relative to controls, with concomitant decreased feed consumption, throughout the treatment period. In addition slightly increased relative liver weight (6%) was noted but is not regarded as toxicity *per se*. Developmental toxicity was evidenced by decreases in foetal body weight and gravid uterine weight. In addition, clear increases in several foetal abnormalities (forelimb flexure, bent clavicle, hindlimb rotation, convoluted ureter, and hydroureter) occurred, which have subsequently been shown to reverse by postnatal day four. The terminal plasma concentrations of sulfoxaflor in both dam and foetal blood were dose-proportional throughout the entire range of dietary exposure concentrations with similar levels between the maternal and foetal blood compartments. Administration of 25 or 150 ppm sulfoxaflor in rodent feed produced no treatment-related maternal toxicity and no indications of embryo/foetal toxicity or teratogenicity.

The LOAEL for maternal toxicity was 1000 ppm (70.2 mg/kg/day) based on decreased body weights and body weight gain. The NOAEL was 150 ppm (11.5 mg/kg/day).

The LOAEL for developmental Toxicity was 1000 ppm (70.2 mg/kg/day) based on decreased number of viable foetuses/litter, decreased foetal weights, convoluted ureter and hydroureter, forelimb flexure and hindlimb rotation, and skeletal alterations including bent clavicles. The NOAEL is 150 ppm (11.5 mg/kg/day).

Study 2a,b, and c: Rabbit developmental toxicity (DAR Ref B.6.6.11.1, 2 and 3):

Study 2a: Rabbit dietary probe study

Report:	Oral gavage developmental toxicity Probe study in New Zealand White Rabbits.
Author:	R. J. Rasoulpour, Ph.D.,K. J. Brooks B.S..
Date of Report:	15 December 2008
Report Identity:	Study ID: 081042
Testing Facility:	Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan, 48674.
GLP	Yes
Test Substance:	XDE-208 (95.6% (wt/wt); as two diastereomers in 48.4/47.4% ratio.
Batch:	E2162-34
Guidelines:	OPPTS 870.3700, OECD 414
Deviations:	None
Acceptable:	Yes

Summary:
The purpose of this study was to make a preliminary evaluation of the maternal toxicity and embryo/foetal lethality potential of sulfoxaflor in New Zealand White rabbits following repeated oral gavage administration. Results from this study will be used to set dose levels for a subsequent gavage developmental toxicity study in rabbits.

Groups of seven time-mated female New Zealand White rabbits were administered sulfoxaflor by gavage at targeted dose levels of 0, 10, 15, 20, or 25 mg/kg/day on gestation days (GD) 7 through 27. In-life parameters evaluated for all groups included clinical observations, body weight, body weight gain, and feed consumption. On GD 28, all surviving rabbits were euthanized and examined for gross pathologic alterations. Liver and kidney weights were recorded, along with the number of corpora lutea, implantations, resorptions, and live/dead foetuses. Blood samples from 5 rabbits/group were taken at 1, 2, 4, 8, and 24 hours after the final dose on GD 27 for analysis of sulfoxaflor levels.

Oral administration of sulfoxaflor by gavage to time-mated New Zealand White rabbits at 25 or 20 mg/kg/day caused severe inanition, and the animals were removed from study on GD 13 or 16, respectively. Animals in the 15 mg/kg/day group had treatment-related body weight loss (14-78 g) upon initiation of dosing (GD 7-10) and an overall decreased mean body weight gain (approximately 39% lower than controls) throughout the dosing period (GD 7-28). There was no maternal toxicity observed at 10 mg/kg/day. There was no indication of embryo/foetal lethality at any dose level. Toxicokinetic analyses on GD 27-28 indicated slow elimination from plasma with a half-life of 14 hours. A 1.5-fold increase in the dose (from 10 to 15 mg/kg/day) resulted in a 1.4-fold increase in the systemic exposure (area under the plasma concentration time-course [AUC] = 236±18 and 332±27 µg h ml-1, respectively).

Based on the results of this study, a dietary probe developmental toxicity study was conducted to select dose levels for the definitive developmental toxicity study.

Study 2b: Rabbit dietary probe study

Report:	Dietary Toxicity/Palatability Prenatal Developmental Probe Study – Rabbits
Author:	R. J. Rasoulpour, Ph.D.,K. J. Brooks B.S..
Date of Report:	14 April 2009
Report Identity:	Study ID: 081121
Testing Facility:	Toxicology & Environmental Research and Consulting, The Dow Chemical

	Company, Midland, Michigan, 48674.
GLP	Yes
Test Substance:	XDE-208 (95.6% (wt/wt); as two diastereomers in 48.4/47.4% ratio.
Batch:	E2162-34
Guidelines:	Non-guideline probe
Deviations:	None
Acceptable:	Yes

SummaryThe purpose of this study was to make a preliminary evaluation of the palatability, maternal toxicity and embryo/foetal lethality potential in New Zealand White rabbits following dietary administration. Results from this study were used to set dose levels and to select the route of exposure for a subsequent developmental toxicity study in rabbits. Groups of five time-mated female New Zealand White rabbits were administered sulfoxaflor by diet at concentrations of 0, 500, or 1000 ppm, which corresponded to time-weighted average doses of 0, 21.7, or 36.6 mg/kg/day, on gestation days (GD) 7 through 28. In-life parameters evaluated for all groups included clinical observations, body weight, body weight gain, and feed consumption. In addition, blood was collected from all surviving rabbits at 1, 2, 4, 8, (GD 27) and 24 hours (GD 28) after the offering of feed on GD 27 to determine blood levels of test material. On GD 28, all surviving rabbits were euthanized and examined for gross abnormalities. Liver and kidney weights were recorded, along with the number of corpora lutea, implantations, resorptions, and live/dead foetuses.

Treatment-related effects in New Zealand White rabbits given 1000 ppm sulfoxaflor by diet consisted of a statistically identified mean body weight loss of 60 g (range +19 to -173 g) after initiation of treatment (GD 7-10) and a 33% decrease in mean body weight gain, relative to controls, throughout the treatment period (GD 7-28). One rabbit in this group had six consecutive days of inanition (GD 9-14) and was euthanized for humane reasons on GD 14. There was no maternal toxicity observed in the 500 ppm rabbits, and there was no indication of embryo/foetal lethality at any dose level.

Toxicokinetic analysis of the time-course plasma concentration of sulfoxaflor from the rabbits exposed through the diet showed that the daily systemic dose (AUC24 h) was dose proportional with constant steady-state plasma concentrations with minimal diurnal fluctuation, compared to the three-fold difference between Cmin and Cmax observed after oral gavage. Dietary administration resulted in a dose corrected AUC24 h of 22 μg h kg-1, which was consistent with previously reported dose corrected values of 20-22 μg h kg-1 following gavage administration. The dietary route afforded a greater applied maximally tolerated dose (1000 ppm = 36.6 mg/kg/day) relative to gavage (15 mg/kg/day caused excessive maternal toxicity). Therefore, the dietary route of administration was chosen for the definitive rabbit developmental toxicity study as it allows for more than twice the applied dose and a corresponding higher AUC24 h as compared to gavage administration.

Study 2c: Main rabbit developmental toxicity study.

The purpose of this study was to evaluate the maternal and developmental toxicity potential of sulfoxaflor in New Zealand White rabbits following dietary administration. Groups of 26 time-mated female rabbits were administered sulfoxaflor at dietary concentrations of 0, 30, 150, or 750 ppm, which corresponded to time-weighted average doses of 0, 1.3, 6.6, or 31.9 mg/kg/day, on gestation days (GD) 7-28. In-life parameters evaluated for all rabbits included: clinical observations, body weight, body weight gain, and feed consumption. Maternal blood was collected for sulfoxaflor analysis from four rabbits/group over a 24-hour period starting on the morning of GD 27, and also at termination on GD 28. Foetal umbilical cord blood was also taken at

termination. All rabbits surviving to GD 28 were euthanized and examined for gross pathologic alterations and changes in liver, kidney, and gravid uterine weight. The number of corpora lutea, uterine implantations, resorptions, and live/dead foetuses were determined. All foetuses were weighed, sexed, and examined for external and visceral alterations. Also, the heads were examined for craniofacial alterations by serial sectioning in approximately one half of the foetuses in each litter, while skeletal examinations were performed on all foetuses.
Animals in the 750 ppm dose group exhibited treatment-related maternal toxicity in the form of decreased feces in 7 of 26 animals, decreased mean body weight gain (55%) from GD 7-13, decreased mean body weight gain (12%) throughout treatment (GD 7-28), and decreased mean feed consumption (8-21%) from GD 7-17. There was no treatment-related maternal toxicity for animals in the 30 or 150 ppm dose groups. There was no treatment-related developmental toxicity in any dose group.

The daily systemic dose of sulfoxaflor on GD 27-28 was dose-proportional as indicated by the near identical mean dose-corrected $AUC_{24\,h}$ values of 18, 19, and 19 µg sulfoxaflor/h/kg^{-1} for animals given 30, 150, and 750 ppm, respectively. Levels of sulfoxaflor in maternal and foetal blood were similar. The daily systemic dose in this dietary study was similar to that measured in prior gavage studies with sulfoxaflor.

Based on these findings, the no-observed-effect level (NOEL) for maternal toxicity was 150 ppm, and the NOEL for developmental toxicity was 750 ppm, the highest dose level tested. In contrast to the rat, sulfoxaflor was not developmentally toxic in the rabbit, despite the achievement of similar maternal and foetal systemic concentrations of sulfoxaflor in both species.
This study is acceptable and satisfies the guideline requirement for a Prenatal Developmental Study – Rabbits; OPPTS 870.3700; OECD 414.

Report:	Dietary Developmental Toxicity Study in New Zealand White Rabbits
Author:	R. J. Rasoulpour, Ph.D.,K. J. Brooks B.S..
Date of Report:	01 September 2009b
Report Identity:	Study ID: 081043
Testing Facility:	Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan, 48674.
GLP	Yes
Test Substance:	XDE-208 (95.6% (wt/wt); as two diastereomers in 48.4/47.4% ratio.
Batch:	E2162-34 TSN003725-0001
Guidelines:	OPPTS 870.3700, OECD 414
Deviations:	None
Acceptable:	Yes

Materials and Methods
The purpose of this study was to evaluate the maternal and developmental toxicity potential of sulfoxaflor in New Zealand White rabbits following dietary administration. Groups of 26 time-mated female rabbits were administered sulfoxaflor at dietary concentrations of 0, 30, 150, or 750 ppm, which corresponded to time-weighted average doses of 0, 1.3, 6.6, or 31.9 mg/kg/day, on gestation days (GD) 7-28. In-life parameters evaluated for all rabbits included: clinical observations, body weight, body weight gain, and feed consumption. Maternal blood was collected for sulfoxaflor analysis from 4 rabbits/group over a 24-hour period starting on the morning of GD 27, and also at termination on GD 28. Foetal umbilical cord blood was also taken at termination. All rabbits surviving to GD 28 were euthanized and examined for gross pathologic alterations and

changes in liver, kidney, and gravid uterine weight. The number of corpora lutea, uterine implantations, resorptions, and live/dead foetuses were determined. All foetuses were weighed, sexed, and examined for external and visceral alterations. Also, the heads were examined for craniofacial alterations by serial sectioning in approximately one half of the foetuses in each litter, while skeletal examinations were performed on all foetuses.

Results

Maternal

Mortality/clinical observations: The only treatment-related clinical observation was decreased faeces observed in 7 of 26 rabbits given 750 ppm. Findings bearing no relationship to treatment included one rabbit (7384) in the 150 ppm group with sporadic decreased/absent faeces from GD 3-21, followed by blood in the cage, soft faeces, and/or faecal and urine soiling on GD 24-25. This rabbit aborted on GD 25 and was euthanized. There were some other sporadic, transient observations (decreased/soft faeces, lacerations/scratches from breeding, cold to the touch, thickened/inflamed skin, excessive hair loss, or decreased urine) that were incidental and not treatment-related.

Body weight/food consumption*:* There were no statistically identified differences in the body weights of any treated groups when compared to control. Treatment-related effects in the 750 ppm group consisted of a decreased mean body weight gain (~50%) upon administration of the treated feed (GD 7-13). The mean body weight gain for the 750 ppm group was comparable to controls throughout the remainder of the study; however, the overall mean body weight gain throughout the dosing period (GD 7-28) was 12% lower in the high-dose group relative to controls (Table 2). There were no treatment-related effects on body weight or body weight gain for animals in the 30 and 150 ppm dose groups

Table 4.11.2.1.Study 2.1 (DAR Table B.6.6.11.3-1): Mean (±SD) Body Weight Gains (g) of Pregnant Females

Time (Day)	Dose Level (ppm) (n=24-26) [a]			
	0	**30**	**150**	**750**
GD 0-7	10.9±66.0	22.9±75.8	14.5±73.7	-1.2±64.1
GD 7-10	28.3±28.1	26.5±30.4	22.7±37.0	9.9±37.4
GD 10-13	74.8±27.9	72.2±33.6	73.7±33.9	41.9±45.5*
GD 13-16	74.2±31.8	94.1±44.7	79.8±63.9	67.9±53.4
GD 16-20	30.8±29.2	27.4±40.3	23.4±43.3	31.3±45.8
GD 20-24	82.4±34.7	65.7±43.2	76.2±41.1	85.2±35.2
GD 24-28	65.7±34.7	54.4±35.2	65.6±32.0	78.6±48.1
GD 7-28	356.3±78.5	340.3±76.4	347.9±82.4	314.9±117.6
GD 0-28	367.2±89.3	363.2±114.5	365.4±111	313.8±127.7

a Change in (n) number were due to abortion of animal # 7384

* Statistically different from control mean by Dunnett's test, α= 0.05

Pre-treatment feed consumption was similar in all groups during the study. There was a treatment-related decrease in mean feed consumption (from 8-21% lower than control) in the 750 ppm dose group from GD 7-17, which correlated with reduced body weight gain during this time period for this dose group (Table 3). Five of the daily feed consumption intervals for the 750 ppm rabbits during this period were statistically identified as lower than controls. In the 30 and 150 ppm dose groups, there were sporadic, statistically-identified, feed consumption intervals that were lower than controls throughout the dosing period, which were deemed spurious and unrelated to treatment due to lack of correlation with decreased body weight gain.

Table 4.11.2.1.Study 2.2 (DAR Table B.6.6.11.3-2.): Mean (±SD) Feed Consumption

(g/animal/day) of Pregnant Females

Time (Day)	Dose Level (ppm) (n=25-26)			
	0	**30**	**150**	**750**
GD 4-5	155.4±4.7	148.7±31.1	153.8±8.7	149.5±15.3
GD 5-6	155.3±6.6	147.5±23.1	153.4±6.7	150.3±15.9
GD 6-7	156.1±5.6	152.9±10.0	152.7±9.1	150.6±16.9
GD 7-8	153.3±7.0	150.4±4.4	153.5±9.7	130.5±28.2*
GD 8-9	153.5±6.2	149.7±5.8	149.1±18.6	139.3±23.3
GD 9-10	154.6±4.0	151.0±5.0*	150.3±9.9	141.7±18.9*
GD 10-11	153.8±5.5	150.2±4.7	147.9±19.9	137.5±34.3
GD 11-12	152.8±7.2	147.6±7.3*	143.9±20.0*	134.3±29.4
GD 12-13	150.1±13.5	144.4±15.1	141.0±30.0	121.7±31.5*
GD 13-14	144.5±25.3	143.4±14.5	134.6±41.3	114.0±41.9*
GD 14-15	151.4±14.2	144.5±17.7*	134.3±44.3*	128.0±44.5*
GD 15-16	152.0±8.7	145.6±21.4	138.5±32.5	135.6±41.7

* Statistically different from control mean by Wilcoxon's test, $\alpha = 0.05$

Gross pathology
There were no treatment-related findings at necropsy.

Caesarean section data
There were no treatment-related effects on pregnancy rates, numbers of corpora lutea, implantations, resorptions, resorptions per litter with resorptions, litter size, or mean pre- or postimplantation loss in animals given sulfoxaflor.

Table 4.11.2.1.Study 2.3 (DAR Table B.6.6.11.3-3): Reproductive and Foetal Observations Made at Necropsy

Dose (PPM)	**0**	**30**	**150**	**750**
Number Bred	26	26	26	26
% Pregnant[a]	25/ 26 (96.2%)	26/ 26 (100%)	25/ 26 (96.2%)	25/ 26 (96.2%)
No. of deaths	0	0	0	0
No. moribund[b]	0	0	0	0
No. aborted	0	0	1	0
No. delivered early	0	0	0	0
Pregnant detected by stain [c]	0/1	0/0	0/1	0/1
No. of litters totally resorbed	0	0	0	0
No. Of litters with viable foetuses	25	26	24	25

No. Of corpora lutea/dam [d]	10.0 ± 1.8	9.0 ± 1.5	9.2 ± 1.6	9.7 ± 1.7
No. Of implantations/dam [d]	9.1 ± 2.2	8.6 ± 1.2	8.6 ± 1.6	9.0 ± 1.7
Mean% preimplantation loss [e]	8.4 ± 14.7	3.4 ± 6.1	5.8 ± 12.5	7.3 ± 8.9
No. Of resorptions/litter [d,g]	0.2 ± 0.6	0.3 ± 0.5	0.3 ± 0.6	0.3 ± 0.6
Resorptions/litter with resorptions [g]	1.7 (5/3)	1.0 (9/9)	1.4 (7/5)	1.1 (8/7)
Mean% postimplantation loss [f]	1.8 ± 5.3	4.0 ± 5.7	3.4 ± 7.2	3.7 ± 6.5
Viable foetuses/litter [d]	8.9 ± 2.1	8.3 ± 1.3	8.3 ± 1.6	8.7 ± 1.7
Foetal weight-males (G)[d]	34.8 ± 4.4	34.9 ± 3.4	34.9 ± 4.1	34.8 ± 3.5
Foetal weight- females [d]	34.9 ± 4.7	33.4 ± 3.4	34.7 ± 3.4	33.9 ± 3.7
Foetal weight – sexes combined (G)[d]	34.6 ± 4.4	34.2 ± 3.0	34.7 ± 3.6	34.4 ± 3.5
Gravid uterine weight (G)[d]	459.4 ± 73.6	419.7 ± 50.2	428.0 ± 65.9	448.8 ± 73.0
Sex ration (M%:F%)	46:54	47:53	50:50	45:55

[a] No. of females with visible implantations/total No. Bred..

[b] Animals were euthanized due to inanition.

[c] No. of females detected as being pregnant after sodium sulphide stain/total No. stained.

[d] Mean ± S.D.

[e] Mean Percent/litter (calculated as [(no. copora lutea - no. implantation)/no. Corpora lutea] X 100

[f] Mean percent/litter (calculated as [(no. implantation – live born pups / no. implantation] X 100

[g] Not statistically analysed.

There were no statistical differences from control at $\alpha = 0.05$.

Developmental/foetal

External examinations: There were no treatment-related external alterations in any dose group. The only foetus (150 ppm group) with an external malformation had flexure of the left forelimb. There were no other foetuses with external malformations in any other dose group.

Visceral examination: There were no treatment-related visceral alterations in any dose group. There was one malformed foetus in the control group that had abnormal course of the jugular vein. There were four malformed foetuses in the 30 ppm group. One foetus had hydronephrosis of the right kidney, two foetuses had missing gallbladders, and one foetus had a missing left testis. There were three malformed foetuses in the 150 ppm group. One foetus had a diaphragmatic hernia, one foetus had a missing gallbladder, and one foetus had a diaphragmatic hernia, hypoplastic lung lobes, hypoplastic heart, and a missing gallbladder. There were two malformed foetuses in the 750 ppm group. Both foetuses had missing gallbladders. The incidence of missing gallbladders in all treated groups was considered unrelated to treatment because of lack of a dose response relationship, and the number of foetuses affected was within the historical control range.

Skeletal examination: There were no treatment-related skeletal alterations in any dose group.

Table 4.11.2.1.Study 2.4 (DAR Table B.6.6.11.3-4.): Incidence of Foetal Alterations

Dose (ppm)		**0**	**30**	**150**	**750**
Number of foetuses (number of litters) examined					
EXTERNAL EXAMINATION		223 (25)	215 (26)	199 (24)	217 (25)
CRANIOFACIAL EXAMINATION		117 (25)	114 (26)	105 (24)	114 (25)
VISCERAL EXAMINATION		223 (25)	215 (26)	199 (24)	217 (25)

VISCERAL EXAMINATION (MALE)		102 (25)	101 (26)	99 (24)	98 (25)
VISCERAL EXAMINATION (FEMALE)		121 (25)	114 (26)	100 (24)	119 (25)
SKELETAL EXAMINATION (HEAD)		106 (25)	101 (26)	94 (24)	103 (25)
SKELETAL EXAMINATION (BODY)		223 (25)	215 (26)	199 (24)	217 (25)
External observations					
		Number affected/total number (% affected)			
FLEXURE FORELIMB+	F	0/223 (0.0)	0/215 (0.0)	1/199 (0.5)	0/217 (0.0)
	L	0/25 (0.0)	0/26 (0.0)	1/24 (4.2)	0/25 (0.0)
Craniofacial observations					
No observations					
Visceral observations					
PARAOVARIAN CYST OVARY	F	1/121 (0.8)	3/114 (2.6)	2/100 (2.0)	1/119 (0.8)
	L	1/25 (4.0)	3/26 (11.5)	2/24 (8.3)	1/25 (4.0)
MISSING TESTIS+	F	0/102 (0.0)	1/101 (1.0)	0/99 (0.0)	0/98 (0.0)
	L	0/25 (0.0)	1/26 (3.8)	0/24 (0.0)	0/25 (0.0)
HEMORRHAGE THYMUS	F	0/223 (0.0)	0/215 (0.0)	0/199 (0.0)	1/217 (0.5)
	L	0/25 (0.0)	0/26 (0.0)	0/24 (0.0)	1/25 (4.0)
MISSING CAUDAL LUNG LOBE	F	13/223 (5.8)	8/215 (3.7)	7/199 (3.5)	14/217 (6.5)
	L	8/25 (32.0)	8/26 (30.8)	6/24 (25.0)	8/25 (32.0)
FUSED LUNG	F	0/223 (0.0)	0/215 (0.0)	0/199 (0.0)	1/217 (0.5)
	L	0/25 (0.0)	0/26 (0.0)	0/24 (0.0)	1/25 (4.0)
HYPOPLASTIC LUNG LOBES+	F	0/223 (0.0)	0/215 (0.0)	1/199 (0.5)[a]	0/217 (0.0)
	L	0/25 (0.0)	0/26 (0.0)	1/24 (4.2)	0/25 (0.0)
DIAPHRAGMATIC HERNIA+	F	0/223 (0.0)	0/215 (0.0)	2/199 (1.0)[a]	0/217 (0.0)
	L	0/25 (0.0)	0/26 (0.0)	2/24 (8.3)	0/25 (0.0)
HYPOPLASTIC HEART+	F	0/223 (0.0)	0/215 (0.0)	1/199 (0.5)[a]	0/217 (0.0)
	L	0/25 (0.0)	0/26 (0.0)	1/24 (4.2)	0/25 (0.0)
MISSING GALL BLADDER+	F	0/223 (0.0)	2/215 (0.9)	2/199 (1.0)[a]	2/217 (0.9)
	L	0/25 (0.0)	1/26 (3.8)	2/24 (8.3)	2/25 (8.0)
RIGHT-SIDED ESOPHAGUS	F	3/223 (1.3)	2/215 (0.9)	2/199 (1.0)	1/217 (0.5)
	L	3/25 (12.0)	2/26 (7.7)	2/24 (8.3)	1/25 (4.0)

ABNORMAL COURSE JUGULAR+	F	1/223 (0.4)	0/215 (0.0)	0/199 (0.0)	0/217 (0.0)
	L	1/25 (4.0)	0/26 (0.0)	0/24 (0.0)	0/25 (0.0)
TORSION STRANGULATION LIVER, MEDIAN LOBE	F	1/223 (0.4)	0/215 (0.0)	0/199 (0.0)	1/217 (0.5)
	L	1/25 (4.0)	0/26 (0.0)	0/24 (0.0)	1/25 (4.0)
TORSION STRANGULATION LIVER, CAUDATE LOBE	F	0/223 (0.0)	1/215 (0.5)	0/199 (0.0)	0/217 (0.0)
	L	0/25 (0.0)	1/26 (3.8)	0/24 (0.0)	0/25 (0.0)
HYDRONEPHROSIS KIDNEY+	F	0/223 (0.0)	1/215 (0.5)	0/199 (0.0)	0/217 (0.0)
	L	0/25 (0.0)	1/26 (3.8)	0/24 (0.0)	0/25 (0.0)
RETROCAVAL URETER	F	2/223 (0.9)	7/215 (3.3)	2/199 (1.0)	3/217 (1.4)
	L	2/25 (8.0)	5/26 (19.2)	1/24 (4.2)	3/25 (12.0)
Skeletal observations					
Dose (ppm)		**0**	**30**	**150**	**750**
DELAYED OSSIFICATION INTERPARIETAL	F	0/106 (0.0)	2/101 (2.0)	1/94 (1.1)	0/103 (0.0)
	L	0/25 (0.0)	2/26 (7.7)	1/24 (4.2)	0/25 (0.0)
DELAYED OSSIFICATION HYOID	F	44/106 (41.5)	40/101 (39.6)	25/94 (26.6)	28/103 (27.2)
	L	16/25 (64.0)	19/26 (73.1)	12/24 (50.0)	14/25 (56.0)
CROOKED HYOID	F	2/106 (1.9)	1/101 (1.0)	2/94 (2.1)	1/103 (1.0)
	L	2/25 (8.0)	1/26 (3.9)	2/24 (8.3)	1/25 (4.0)
DELAYED OSSIFICATION STERNEBRAE	F	60/223 (26.9)	72/215 (33.5)	57/199 (28.6)	40/217 (18.4)
	L	21/25 (84.0)	23/26 (88.5)	19/24 (79.2)	14/25 (56.0)
FUSED STERNEBRAE	F	8/223 (3.6)	1/215 (0.5)	1/199 (0.5)	1/217 (0.5)
	L	4/25 (16.0)	1/26 (3.8)	1/24 (4.2)	1/25 (4.0)
EXTRA SITE OF OSSIFICATION STERNEBRAE	F	1/223 (0.4)	1/215 (0.5)	2/199 (1.0)	0/217 (0.0)
	L	1/25 (4.0)	1/26 (3.8)	2/24 (8.3)	0/25 (0.0)
IRREGULAR PATTERN OF OSSIFICATION STERNEBRAE	F	3/223 (1.3)	1/215 (0.5)	3/199 (1.5)	0/217 (0.0)
	L	3/25 (12.0)	1/26 (3.8)	3/24 (12.5)	0/25 (0.0)
DELAYED OSSIFICATION TALUS	F	4/223 (1.8)	0/215 (0.0)	1/199 (0.5)	0/217 (0.0)
	L	2/25 (8.0)	0/26 (0.0)	1/24 (4.2)	0/25 (0.0)
DELAYED OSSIFICATION PUBIS	F	5/223 (2.2)	4/215 (1.9)	8/199 (4.0)	3/217 (1.4)
	L	3/25 (12.0)	4/26 (15.4)	5/24 (20.8)	3/25 (12.0)

Total malformed					
Dose (ppm)		**0**	**30**	**150**	**750**
TOTAL MALFORMED EXTERNAL	F	0/223 (0.0)	0/215 (0.0)	1/199 (0.5)	0/217 (0.0)
	L	0/25 (0.0)	0/26 (0.0)	1/24 (4.2)	0/25 (0.0)
TOTAL MALFORMED CRANIOFACIAL	F	0/117 (0.0)	0/114(0.0)	0/105 (0.0)	0/114 (0.0)
	L	0/25 (0.0)	0/26 0.0)	0/24 (0.0)	0/25 (0.0)
TOTAL MALFORMED VISCERAL	F	1/223 (0.4)	3/215 (1.4)	3/199 (1.5)	2/217 (0.9)
	L	1/25 (4.0)	2/26 (7.7)	3/24 (12.5)	2/25 (8.0)
TOTAL MALFORMED VISCERAL (FEMALE GONADS)	F	0/121 (0.0)	0/114 (0.0)	0/100 (0.0)	0/119 (0.0)
	L	0/25 (0.0)	0/26 (0.0)	0/24 (0.0)	0/25 (0.0)
TOTAL MALFORMED VISCERAL (MALE GONADS)	F	0/102 (0.0)	1/101(1.0)	0/99 (0.0)	0/98 (0.0)
	L	0/25 (0.0)	1/26 (3.8)	0/24 (0.0)	0/25 (0.0)
TOTAL MALFORMED SKELETAL	F	0/223 (0.0)	0/215 (0.0)	0/199 (0.0)	0/217 (0.0)
	L	0/25 (0.0)	0/26 (0.0)	0/24 (0.0)	0/25 (0.0)
TOTAL MALFORMED SKELETAL (HEAD)	F	0/106 (0.0)	0/101 (0.0)	0/94 (0.0)	0/103 (0.0)
	L	0/25 (0.0)	0/26 (0.0)	0/24 (0.0)	0/25 (0.0)
TOTAL MALFORMED OVERALL	F	1/223 (0.4)	4/215 (1.9)	4/199 (2.0)	2/217 (0.9)
	L	1/25 (4.0)	3/26 (11.5)	3/24 (12.5)	2/25 (8.0)

F = Foetuses; L = Litters
\+ Considered a malformation
@ Not statistically analyzed
a Malformations denoted with the same superscript were noted in a single foetus

Toxicokinetics

Summary data describing systemic exposure of sulfoxaflor to rabbits exposed through diet for 21 days and sampled 1, 8 and 24 h after the start of the light cycle on GD 27 are presented in Table B.6.6.11.3-5. There was a dose-proportional increase in the daily systemic dose (AUC24 h), as evidenced by mean values of 20.5, 107, and 598 µg h kg-1 for the 30, 150, and 750 ppm groups, respectively. Dose proportionality was also apparent from the near identical mean dose-corrected AUC24 h values, which were 18, 19, and 19 µg h kg-1, respectively.

Table 4.11.2.1.Study 2.5 (DAR Table B.6. 6.11.3-5): Toxicokokinetics Results of Systemic Exposure of Sulfoxaflor to Rabbit for 21 days via Diet

Dose (mg/kg/day)		**AUC_{24h} (µg h/ml)**		**Dose corrected AUC_{24h}**		**Plasma elimination $t_{1/2}$ (h)**	
Mean	SD	Mean	SD	Mean	SD	Mean	SD
1.15	0.209	20.54	2.82	18.0	1.75	ND	ND
5.59	1.29	107.13	19.76	19.3	1.07	ND	ND
31.54	0.737	598.84	58.22	19.0	1.43	ND	ND

A comparison of the daily systemic dose (AUC24 h) of sulfoxaflor in rabbits (oral gavage or dietary administration) with a developmental toxicity study in rats (dietary administration) is presented in Table B.6.6.11.3-6. The daily systemic doses for the low-, mid-, and high-dose levels in the definitive rat developmental toxicity study were 20.2, 119, and 846 µg h kg-1, respectively, while the daily systemic dose values for the low-, mid-, and high-dose levels in the present rabbit developmental toxicity were 20.5, 107, and 599 µg h kg-1, respectively. Comparison of daily systemic dose at the no-observed-adverse-effect level (NOAEL) and low-observed-adverse-effect level (LOAEL) for developmental toxicity shows an internal dose of 119 and 846 µg h kg -1 at the rat NOAEL and LOAEL, respectively, 599 µg h kg-1 at the rabbit NOAEL. These data demonstrate that with similar internal dose levels, the rat, but not the rabbit, is sensitive to sulfoxaflor induced developmental toxicity.

Table 4.11.2.1.Study 2.6 (DAR Table B.6.6.11.3-6): Comparison of Toxicokinetics Results of Sulfoxaflor in Rabbits and Rats Exposed via Gavage and Diet for Different Duration – Systemic Exposure

Daily Dose of Sulfoxaflor and Plasma AUC_{24h} in Rabbits and Rats			
Dose (mg/kg/day) Mean ± SD	**AUC (µg h ml^{-1})[a] Mean ± SD**	**Dose Corrected AUC[b] Mean ± SD**	**Plasma Elimination $t_{1/2}$ (h) Mean ± SD**
(A) Rabbit **Daily Dietary Dose to Rabbits for 20 Days (Diet: Definitive Study; current study)**			
1.15±0.209	20.5±2.83	18.0±1.75	ND
5.59±1.29	107±19.8	19.3±1.07	ND
31.5±0.737	599±58.2	19.0±1.43	ND
Daily Dietary Dose to Rabbits for 20 Days (Diet: Palatibility Study; MRID 47832065)			
20.0±2.77	439±37.0	21.9±1.85	ND
35.2±5.06	776±70.4	22.0±2.00	ND
Daily Gavage Dose to Rabbits for 20 Days (Gavage: Probe Study; MRID 47832139)			
10.0±---	235±20.9	23.5±2.09	14.1±1.5
15.0±---	332±26.9	22.1±1.79	13.5±1.2
Daily Gavage Dose to Rabbits for 20 Days (Gavage: Range-finding Study; MRID 47832139)			
10.0±---	159±---	15.9±---	14.8±---
20.0±---	404±---	20.2±---	24.8±---
30.0±---	659±---	22.0±---	35.2±---
(B) Rats **Daily Dietary Dose to Rats for 16 Days (Diet: Developmental Study; MRID 47832140)**			
1.60±0.138	20.2±2.06	12.7±1.29	---
9.29±0.992	119±20.8	12.8±2.24	---
64.2±7.41	846±130	13.2±2.03	---

a=Rat AUC values calculated from single-time point determinations X 24 hr
b= Dose corrected AUC = AUC / dose
ND = Not calculated due to no drop in plasma concentration after the removal of dose

Conclusions

Animals in the 750 ppm dose group exhibited treatment-related maternal toxicity in the form of decreased faeces in 7 of 26 animals, decreased mean body weight gain (55%) from GD 7-13, decreased mean body weight gain (12%) throughout treatment (GD 7-28), and decreased mean feed consumption (8-21%) from GD 7-17. There was no treatment-related maternal toxicity for animals in the 30 or 150 ppm dose groups. There was no treatment-related developmental toxicity in any dose group.

The daily systemic dose of sulfoxaflor on GD 27-28 was dose-proportional as indicated by the near identical mean dose-corrected AUC24 h values of 18, 19, and 19 µg sulfoxaflor/h/kg-1 for animals

given 30, 150, and 750 ppm, respectively. Levels of sulfoxaflor in maternal and foetal blood were similar. The daily systemic dose in this dietary study was similar to that measured in prior gavage studies with sulfoxaflor.
Based on these findings, the no-observed-effect level (NOEL) for maternal toxicity was 150 ppm (6.6 mg/kg/day) and the maternal LOAEL was 750 ppm (31.9 mg/kg/day) based on decreased body weight and weight gain. The NOEL for developmental toxicity was 750 ppm (31.9 mg/kg/day), the highest dose level tested.
Sulfoxaflor was not toxic to development in the rabbit, (in contrast to the rat), despite the achievement of similar maternal and foetal systemic concentrations of sulfoxaflor in both species.

Study 3: Rat Developmental Neurotoxicity study (DAR Ref B.6.7.5):

In a developmental neurotoxicity study in rats sulfoxaflor (purity 95.6%; Lot # E2162-32) was offered on a continuous basis in the diet to 3 groups of 25 bred female Crl:CD(SD) rats daily from gestation day 6 through lactation day 21. Target test substance concentrations were 25, 100, and 400 ppm, which corresponded to predicted dosage levels of 2, 8, and 32 mg/kg/day, respectively. Actual overall mean test substance consumption in the 25, 100, and 400 ppm groups was 1.8, 7.1, and 27.7 mg/kg/day through gestation and 1.9, 7.6, and 29.8 mg/kg/day through lactation, respectively. A concurrent control group composed of 25 bred females received the basal diet on a comparable regimen. Dams were approximately 13 weeks of age at the beginning of test diet exposure.

There were no test substance-related mortalities in the dams during the study. There were no test substance-related clinical findings noted during the daily examinations. Detailed clinical observation parameters, as well as maternal body weights and food consumption during gestation and lactation were unaffected by test substance exposure.

There were no test substance-related differences noted between groups when comparing the mean length of gestation, the process of parturition, and internal macroscopic pathologic findings. The mean numbers of former implantation sites and unaccounted for sites, as well as maternal kidney and liver weights were similar across groups.

There were no test substance-related effects on maternal parameters in this study. Therefore, the no-observed-adverse-effect-level (NOAEL) for systemic toxicity and maternal reproductive toxicity of sulfoxaflor when administered orally in the diet was 400 ppm (equivalent to 28.8 mg/kg/day).

There were no test substance-related effects on the mean number of pups born, live litter size, or the percentage of males at birth at any maternal exposure level. However, offspring toxicity was expressed at 400 ppm by a statistically significant reduction in postnatal survival from birth to PND 4 compared to the control group. Furthermore, mean pup body weights in the 400 ppm group were 11.8% and 6.5% lower than the control group at birth (PND 1) and on PND 4, respectively. The reduced pup body weights resulted in a statistically significant delay in surface righting response for pups in the 400 ppm group. Pup body weights in the 400 ppm group did not differ from the control group values on PND 7 or later time points. The decrease in postnatal survival at 400 ppm is consistent with results from a previous probe reproduction study, in which dietary exposures of 500 and 1000 ppm resulted in decreased pup survival. The high dose level of 400 ppm in this study was based on the treatment-related decrease in survival observed in the probe study. Postnatal survival and pup body weights and body weight gains in the 25 and 100 ppm groups were unaffected by maternal test substance exposure. The age of attainment of surface righting response in the 25 and 100 ppm groups and eye opening in the 25, 100, and 400 ppm groups were similar to the control group. The attainment of sexual developmental landmarks (balanopreputial separation and vaginal patency) were unaffected by maternal test substance exposure.

No remarkable clinical observations or macroscopic findings were noted for offspring at any exposure level. No test substance-related effects were observed with respect to detailed clinical observations, locomotor activity, auditory startle response, and learning and memory. Furthermore, there were no test substance-related effects on brain weights, measurements, and morphometric parameters or histopathology of the brain and/or central and peripheral nervous systems for offspring on PND 21 and 72.

Offspring LOAEL is based on the reduction in postnatal survival, decreased pup body weights, delayed righting reflex, decreased brain weight in males, and altered brain length in males and females, at 400 ppm. The NOAEL for neonatal toxicity was 100 ppm (equivalent to 7.4 mg/kg/day).

The study is acceptable.

Report:	A Dietary Developmental Neurotoxicity Study of XDE-208 in Rats
Author:	Beck, M.J.
Date of Report:	June 2010
Report Identity:	MRID 47832133,
Testing Facility:	WIL Research Laboratories, LLC, Ashland, OH, USA.
GLP	Yes
Test Substance:	XR-208 (purity 95.6%; Lot # E2162-32)
Batch:	E2162-32
Guidelines:	U.S. EPA OPPTS 870.6300 OECD 426
Deviations:	None
Acceptable:	Yes

Materials and Methods:
A developmental neurotoxicity study in rats was carried out (MRID 478321333), sulfoxaflor (purity 95.6%; Lot # E2162-32) was offered on a continuous basis in the diet to 3 groups of 25 bred female Crl:CD(SD) rats daily from gestation day 6 through lactation day 21. Target test substance concentrations were 25, 100, and 400 parts per million (ppm), which corresponded to predicted dosage levels of 2, 8, and 32 mg/kg/day, respectively. Actual overall mean test substance consumption in the 25, 100, and 400 ppm groups was 1.8, 7.1, and 27.7 mg/kg/day through gestation and 1.9, 7.6, and 29.8 mg/kg/day through lactation, respectively. A concurrent control group composed of 25 bred females received the basal diet on a comparable regimen. Dams were approximately 13 weeks of age at the beginning of test diet exposure.

Study Protocol:

The study was carried out in accordance with OECD Guideline 426

Results

Maternal Animals

Pregnancy status: The pregnancy rates in the control, 25, 100, and 400 ppm groups were 100%. However, 1 female (no. 49879) in the 100 ppm group failed to deliver and a pregnancy status was inadvertently not determined. Because the female that failed to deliver was most likely nongravid, it is assumed that the pregnancy status in the 100 ppm group was 96.0%.

Mortality and clinical signs: All dams survived to the scheduled necropsies. Female no. 49902 in

the 400 ppm group had total litter loss on PND 2. Total litter loss was also noted for 2 females (nos. 49934 and 49916) in the control group on lactation days 0 and 9, respectively. No test substance-related clinical findings were noted during the daily examinations at any exposure level. Findings noted in the test substance-exposed groups, including hair loss, scabbing, and red material on various body surfaces, occurred infrequently, at similar frequencies in the control group, and/or in a manner that was not exposure-related.

Detailed clinical observations: No test substance-related findings were observed in maternal animals at the detailed clinical observations. A significantly ($p<0.05$) lower number in the 100 ppm group were sitting or standing normally on gestation day 15 compared to the control group (8 vs. 16 animals); however, in the absence of a dose response, this decrease in normal body posture was not considered test substance-related. No other remarkable differences were apparent between the control and test substance-exposed groups when the detailed clinical observation data were evaluated on gestation days 10 and 15 and lactation days 10 and 21.

Body weight: Mean maternal body weights and body weight gains were unaffected by test substance exposure during gestation. Differences between the control, 25, 100, and 400 ppm groups were slight and not statistically significant.

Mean maternal body weights and body weight gains were unaffected by test substance exposure during lactation. A significant ($p=0.012$) treatment-by-time interaction was noted in the analysis of mean body weights for the 100 ppm group; however, because there was no effect on mean body weights in the 400 ppm group, the difference was not considered test substance-related. Furthermore, the increase in mean body weights in the 100 ppm group were minimal (≤4.1% difference from the control group across the intervals measured) and not biologically meaningful.

Food consumption: Mean food consumption, evaluated as g/animal/day, was unaffected by test substance exposure during gestation. A significant ($p=0.031$) treatment-by-time interaction was noted for mean food consumption. When subsequent pairwise comparisons were conducted, the treatment-by-time interaction was significant ($p=0.005$) at 400 ppm. However, because the differences in mean food consumption between the control and 400 ppm groups were slight (1-3 g/animal/day) and in the absence of any effects on mean body weight gains during gestation at this exposure level, the statistically significant treatment-by-time interaction at 400 ppm was not considered test substance-related.

Mean food consumption was unaffected by test substance exposure during lactation. Differences between the control, 25, 100, and 400 ppm groups were slight and not statistically significant.

Test substance intake: The average quantities of sulfoxaflor consumed during the maternal generation are presented in Table 6.

Table 4.11.2.1.Study 3.1 (DAR Table B.6.7.2/1-1): Mean Test Substance Intake mg/kg/day

Mean Test Substance Intake mg/kg/day		
Theoretical Dietary Level	**Gestation**	**Lactation**
25 ppm (2 mg/kg/day)	1.8	1.9
100 ppm (8 mg/kg/day)	7.1	7.6
400 ppm (32 mg/kg/day)	27.7	29.8
a = Summation of mean test substance consumption for the specified interval		

NUMBER OF DAYS OR INTERVALS ASSESSED

.

Gestation length and parturition: No test substance-related effects were noted on mean gestation lengths or the process of parturition at any exposure level. Mean F_0 gestation lengths in the test substance-exposed groups were similar to the control group value. Differences were slight and not statistically significant. The mean gestation lengths in the 25, 100, and 400 ppm groups were 21.6, 21.9, and 21.6 days, respectively, compared to a mean gestation length of 21.9 days in both the concurrent control group and WIL historical control data. No signs of dystocia were noted at any exposure level.

Maternal postmortem results:

a) Gross Pathology: No test substance-related internal findings were observed at any exposure level. The only macroscopic finding noted was dark red contents in the stomach for 1 female (no. 49902) in the 400 ppm group; this female had total litter loss on lactation day 2.

At the lactation day 21 necropsy, no test substance-related effects were observed on the number of former implantation sites and the number of unaccounted-for sites. The differences between the control and test substance-exposed groups were slight and not statistically significant.

b) Organ Weights: There were no test substance-related effects on maternal kidney or liver weights (absolute or relative to final body weight) at any exposure level. Differences in absolute weight between the control and test substance-exposed groups were not statistically significant.

Litter Data

PND 0 litter data and postnatal survival: Test substance-related effects on postnatal survival were noted at 400 ppm. PND 0-1 and 1-4 (pre selection), postnatal survival in the 400 ppm group (86.9% and 87.2% per litter, respectively) was lower than the concurrent control group (99.5% and 99.8% per litter, respectively); the difference was significant ($p<0.05$) during PND 0-1. As a result, postnatal survival in this group (76.5% per litter) from birth to PND 4 (pre-selection) was significantly ($p<0.01$) lower than the concurrent control group value (93.0% per litter) and the value was below the minimum mean value in the WIL historical control data (83.8% per litter). However, postnatal survival in the 400 ppm group was similar to the concurrent control group throughout the remainder of the pre-weaning period from PND 4 [post-selection] to PND 21.

There was some variance in the survival of litters in the concurrent control group at 2 time points during the pre-weaning period. Three litters in the control group largely contributed to this increased variance in pup survival: on PND 0, litter nos. 49881 and 49934 had 70.6% and 0.0% survival, respectively, and during PND 7-14, litter no. 49916 had 0.0% survival (total litter loss on PND 9). Despite the reduced survival in the 3 aforementioned litters, mean postnatal survival values in the concurrent control group were still within the range of values in the WIL historical control data and greater than the values at 400 ppm, indicating a treatment-related effect on pup survival at 400 ppm.

The mean number of pups born, live litter size, and percentage of males per litter at birth in the 25, 100, and 400 ppm groups, and postnatal survival in the 25 and 100 ppm groups were unaffected by maternal exposure to the test diet. Differences from the control group were slight, were not statistically significant, and/or did not occur in an exposure-related manner.

Mean litter size and viability (survival) results from pups during lactation are summarized from the report in Table 7.

Table 4.11.2.1.Study 3.2 (DAR Table B.6.7.2/1-1.) Litter size and viability

Litter size and viability				
Observation	**Dose (ppm)**			
	Control (0)	**25**	**100**	**400**
Offspring Generation				
Mean implantation sites	16.2±1.44/24	15.7±2.27/25	16.4±2.30/24	15.9±1.62/24
Total number born (mean±SD)	14.8±3.14/25	15.2±2.08/25	15.3±2.14/24	15.2±1.75/25
Number born live (mean±SD)	14.2±3.84/25	15.0±2.09/25	15.1±2.12/24	14.9±1.87/25
Sex Ratio Day 0 (% males)	48.7±14.08/25	52.2±12.08/25	51.0±10.94/24	51.3±8.89/25
Mean litter size (%): (mean±SD/N) relative to number born				
Day 0	93.8±20.48/25	99.2±2.87/25	98.7±3.2/24	98.3±3.88/25
Day 1	99.5±1.76/24	98.9±2.51/25	96.0±7.82/24	86.9+±19.22/25
Day 4 [b]	99.8±1.20/24	99.8±1.11/25	98.4±3.77/24	87.2±24.82/25
Day 4-7[c]	99.5±2.61/23	100±0.0/25	99.5±2.61/23	100±0.0/21
Day 7-14	95.7±20.85/23	100±0.0/25	100±0.0/23	99.4±2.73/21
Day 14-21	100±0.00/22	100±.0.0/25	100±0.0/23	99.4±2.73/21
Live birth index (%)	96.0	98.8	98.7	98.0
Viability index (%)	93.0	97.9	93.2	76.5**
Lactation index (%)	95.1	100.0	99.5	98.8

SD = standard deviation N = Number of litters
[b] Before standardization (culling).
[c] After standardization (culling).
** Statistically different from control, p<0.01
Source: Tables 22, 24-25, pp. 171, 174-176 and Tables A23-A24, pp. 790-782 of the study report.

General physical condition: Pups (litters) that were found dead or euthanized *in extremis* numbered 24(8), 6(5), 17(11), and 59(15) in the control, 25, 100, and 400 ppm groups, respectively. Two (2), 2(2), 8(5), and 36(12) pups (litters) in the same respective groups were missing and presumed to have been cannibalized. In addition, malrotation of the left forelimb was noted for 2 pups in the same litter in the 400 ppm group during the week prior to weaning (on PND 14, 17, and/or 21); this observation was not apparent on PND 1, 4, 7, or 11 for either of these pups, both of which survived to the scheduled euthanasia on PND 21. The general physical condition of all F_1 pups in the 25 and 100 ppm groups was unaffected by maternal test substance exposure.

Body weight: Mean pup birth weights (PND 1) in the 400 ppm group were 11.8% lower than the control group. Mean pup weights in this group remained lower (6.5%) than the control group on PND 4 (pre- and post-culling), but were similar to the control group during the remainder of the pre-weaning period (PND 7-21). The recovery in pup body weights was likely due to the early deaths (prior to PND 4) of pups that generally had smaller body weights in the litters. The treatment-by-time interaction at 400 ppm was significant ($p<0.001$) during both the pre-culling and post-culling periods; however, with the exception of the post-culling weight on PND 4, pup body weights in the 400 ppm group were similar to the control group (i.e., within 5%) during the post

culling period (PND 4-21). Mean body weight gains in the 400 ppm group were similar to the control group throughout the pre-weaning period.

Mean pup body weights and body weight changes in the 25 and 100 ppm groups throughout the postnatal period were unaffected by maternal exposure to the test substance. The significant ($p<0.001$) treatment-by-time interactions for mean pup body weights at 25 and 100 ppm during the post-culling period were attributed to sporadic, slightly higher (up to 4.1%) mean body weights that were not considered toxicologically important. Selected mean pup body weight data are presented in Table 8.

Table 4.11.2.1.Study 3.3 (DARTable B.6.7.2/1-2): Mean (±SD) pre-weaning pup body weights (g)

Mean (±SD) pre-weaning pup body weights (g)								
Postnatal day	**Dose (ppm)**							
	0	**25**	**100**	**400**	**0**	**25**	**100**	**400**
	Males				**Females**			
1	7.00 (±0.578)	6.95 (±0.546)	6.93 (±0.570)	6.20 (±0.709)	6.59 (±0.527)	6.52 (±0.513)	6.43 (±0.510)	5.90 (±0.694)
4 [a]	9.53 (±1.27)	9.37 (±0.923)	9.49 (±1.12)	8.78 (±1.17)	8.95 (±1.16)	8.82 (±0.887)	8.84 (±0.951)	8.39 (±1.07)
7 [b]	12.16 (±3.71)	12.17 (±3.97)	12.31 (±3.98)	11.50 (±3.85)	11.39 (±3.45)	11.42 (±3.67)	11.58 (±3.87)	11.06 (±3.77)
11	22.35 (±2.19)	22.98 (±2.19)	22.92 (±1.73)	22.02 (±2.93)	20.87 (±2.35)	21.28 (±2.40)	21.94 (±1.82)	21.53 (±2.56)
17	35.74 (±3.26)	36.51 (±3.05)	35.74 (±3.35)	33.43 (±4.26)	34.00 (±3.09)	34.33 (±3.53)	34.56 (±3.16)	33.02 (±3.84)
21	44.81 (±4.16)	47.30 (±4.69)	46.15 (±5.23)	44.21 (±6.41)	43.03 (±4.72)	44.14 (±4.68)	44.23 (±4.47)	43.41 (±5.49)

[a] Before standardization (culling).

b After standardization (culling on day 4).

Source: Table 27-28, pp. 181-186 and Table A26-A27, pp. 1093-1166 in the study report.

Table B.6.7.2/1-3. Mean (±SD) post-weaning pup body weights (g)

Mean (±SD) post-weaning pup body weights (g)								
Postnatal day	**Dose (ppm)**							
	Control	**25**	**100**	**400**	**Control**	**25**	**100**	**400**
	Males				**Females**			
35	139.8 (±15.3)	143.3 (±15.7)	143.3 (±18.1)	127.05 (±23.2)	119.7 (±13.0)	120.3 (±12.7)	121.65 (±12.5)	121.35 (±12.0)
49	266.8 (±26.6)	271.7 (±23.3)	272.95 (±30.0)	249.95 (±36.7)	184.6 (±14.2)	182.3 (±19.7)	185.5 (±18.0)	185.85 (±17.1)
72	421.3 (±40.8)	426.8 (±36.9)	425.5 (±44.0)	394.6 (±44.0)	253.2 (±20.6)	251.8 (±29.5)	253.85 (±21.8)	249.7 (±25.8)

Source: Table 37-39, pp. 196-201 and Table 36, pp. 1353-1372 in the study report.

Offspring postmortem results:

Necropsies of Pups Found Dead or Euthanized *in Extremis*: The numbers of pups (litters) found dead or euthanized *in extremis* from PND 0 through the selection for evaluation subsets numbered 24(8), 6(5), 17(11), and 59(15) in the control, 25, 100, and 400 ppm groups, respectively. No internal for gross pathological findings that could be attributed to parental exposure to the test substance were noted at the necropsies of pups that were found dead or euthanized *in extremis*.

Necropsies of Pups Not Selected for Neuropathological Evaluation (PND 21) and Pups Euthanized due to Sex Ratio Criteria not Met (PND 4): No internal findings that could be attributed to maternal exposure to the test substance were noted at the necropsy of pups euthanized on PND 4 due to sex ratio criteria not met or on PND 21. Aside from the presence of milk in the stomach, the only internal finding noted in the test substance-exposed groups was a dilated right renal pelvis for pup no. 49951-02 in the 25 ppm group. A dilated right renal pelvis, as well as a pale kidney, was also noted for pup no. 49937-02 in the control group. No other internal findings were noted.

Developmental Landmarks

Surface righting response: A significant ($p<0.001$) delay in the mean age of attainment of surface righting response was noted in the 400 ppm pups (6.3 days) when compared to the concurrent control group value (5.3 days). The mean age of attainment at 400 ppm was also greater than the maximum mean age for males and females in the WIL historical control data (5.1 and 5.3 days, respectively). This test substance-related effect on surface righting response corresponded to reduced mean pup body weights that were noted on PND 1 and 4 in the 400 ppm group. The delay in attainment of surface righting response at 400 ppm is an indication of a slight developmental delay associated with reduced body weight at this exposure level, and not a specific neurobehavioral deficit.

Surface righting response for the pups in the 25 and 100 ppm groups was not affected by F_0 maternal exposure to the test substance. The mean age of attainment was 5.2 days for pups in both the 25 and 100 ppm groups compared to 5.3 days in the control group; differences were not statistically significant.

Eye opening: Eye opening in the pups was not affected by maternal exposure to the test substance. The mean ages of attainment were 14.8, 15.1, 14.8, and 14.9 days for pups in the control, 25, 100, and 400 ppm groups, respectively. The test substance-exposed group values were not statistically

significantly different from the control group values.

Balanopreputial separation: Mean ages of attainment of balanopreputial separation and mean body weights at the age of attainment were unaffected by maternal exposure to the test substance. The mean ages of attainment of balanopreputial separation were 46.3, 46.1, and 47.6 days in the 25, 100, and 400 ppm groups, respectively, compared to 46.9 days in the concurrent control group; all values were within the WIL historical control data range (42.3 to 49.0 days of age). Mean body weights at the age of attainment were 247.1 g, 245.3 g, and 236.4 g in the same respective groups compared to 247.1 g in the concurrent control group and 228.8 g in the WIL historical control data. None of the differences from the control group were statistically significant.

Vaginal patency: Mean ages of attainment of vaginal patency and mean body weights at the age of attainment were unaffected by maternal exposure to the test substance. The mean ages of attainment of vaginal patency were 32.9, 32.7, and 32.6 days in the 25, 100, and 400 ppm groups, respectively, compared to 32.7 days in the control group. Mean body weights at the age of attainment were 106.0 g, 105.1 g, and 104.5 g in the same respective groups compared to 103.9 g in the control group. None of the differences from the control group were statistically significant.

Offspring

Mortality and clinical signs: Following weaning of the pups, male no. 49937-05 in the control group and female no. 49873-10 in the 400 ppm group were found dead on PND 22 and 28, respectively. No remarkable clinical observations or macroscopic findings were noted for either of these animals. Because of the mortality in the control group, the single death in the 400 ppm group was not considered test substance-related. All other offspring survived to the scheduled necropsies.

No test substance-related clinical findings were noted during the weekly examinations of the pups. Findings noted in the test substance-exposed groups, including hair loss on the forelimbs and red material around the nose, mouth, and eyes, occurred infrequently, at similar frequencies in the control group, and/or in a manner that was not exposure-related.

Body weights: Mean weekly post-weaning body weights and body weight gains in the offspring in the 25, 100, and 400 ppm groups were unaffected by maternal exposure to the test substance. Differences from the control group were slight and not statistically significant.

Detailed clinical observations: No consistent exposure-related trends were noted when detailed clinical observation data were evaluated for pups on PND 4, 11, 21, 35, 45, and 60. Findings in the test substance-exposed groups were noted infrequently, similar to the control group, and/or in a manner that was not exposure-related. On PND 60, red deposits around the nose were noted for 6 males in the control group compared to only a single male in the 400 ppm group; the difference was significant ($p<0.05$). However, a decrease in the number of males with red deposits around the nose is not considered toxicologically important. A significantly ($p<0.05$) higher number of females in the 25 ppm group were noted with alert body posture on PND 60 compared to the control group (9 vs. 2 females). In the absence of a dose response, the increased number of alert females in the 25 ppm group was not considered test substance-related. Backing was observed for 1 male in the 25 ppm group on PND 45, and 1-2 males in the 100 ppm group on PND 11, 21, and 45. Because backing was also noted for 1 control group female on PND 21 and was not observed in the 400 ppm group, the sporadic occurrences of backing in the 25 and 100 ppm groups were not considered test substance-related.

Table 4.11.2.1.Study 3.4 (DAR Table B.6.7.2/1- 4): Functional observational battery results (incidence)

Functional observational battery results (incidence)				
Observation	**Dose (ppm)**			
	0	**25**	**100**	**400**
Males				
Red deposits around nose				
-PND 4	--	--	--	--
-PND 11	--	--	--	--
-PND 21	--	--	--	--
-PND 35	--	--	--	--
-PND 45	--	--	--	--
-PND 60	6	--	--	1*[#]
Backing				
-PND 4	--	--	--	--
-PND 11	--	--	1[##]	--
-PND 21	--	--	1[##]	--
-PND 35	--	--	2[##]	--
-PND 45	--	1	--	--
-PND 60	--	--	--	--
Females				
Alert body posture				
-PND 4	--	--	--	--
-PND 11	--	--	--	--
-PND 21	--	--	--	--
-PND 35	--	--	--	--
-PND 45	--	--	--	--
-PND 60	2	9*[##]	--	--
Backing				
-PND 4	--	--	--	--
-PND 11	--	--	--	--
-PND 21	1	--	--	--
-PND 35	--	--	--	--
-PND 45	--	--	--	--
-PND 60	--	--	--	--

-- = Observation did not occur.
[#] Not considered toxicologically important.
[##] Not considered to be test related.
N = 10/sex/dose
Source: Table 49-50, pp. 296-299 in the study report.

Locomotor activity: Locomotor activity patterns (total activity counts) in pups were unaffected by maternal test diet exposure at all exposure levels when evaluated on PND 13, 17, 21, and 61. Values obtained from the 6 subintervals evaluated (0-10, 11-20, 21-30, 31-40, 41-50, and 51-60 minutes) and the overall 60 minute test session values were generally comparable to the concurrent control values and within the WIL historical control data ranges. No remarkable shifts in the pattern of adaptation occurred in any of the test substance-exposed groups.

On PND 13, mean total counts in the 400 ppm group during the individual subintervals (0-10, 11-20, 21-30, 31-40, 41-50 and 51-60 minutes) were higher than the control group values. As a result, mean total counts in the 400 ppm group during the overall test session on PND 13 were 52.1% higher than the control group. However, these increases in motor activity were primarily attributed to 2 littermates (male no. 49861-07 and female no. 49861-10) in this group that had abnormally high total counts during the PND 13 test session. When these 2 outlier animals were excluded, mean total counts in the 400 ppm group for the overall test session on PND 13 were only 19.7% higher than the control group. Furthermore, in the absence of statistical significance across the treatment groups, the higher mean total counts noted in the 400 ppm group on PND 13 were not

considered test substance-related.

On PND 17, there was a significant (p=0.029) treatment-by-time intereaction for mean total counts. When subsequent pairwise comparisons were conducted, significance (p=0.007) was achieved at the low- and mid-exposure levels (25 and 100 ppm, respectively). However, statistical significance was not achieved at the high-exposure level (400 ppm), indicating the absence of a dose response. Furthermore, the significance achieved at 25 and 100 ppm was primarily the result of faster adaptation in these groups when compared to the control group, as mean total counts were slightly higher (15.9% to 21.6%) than the control group during the first 10-minute subinterval (0-10 minutes), but were approximately 18% to 27% lower than the control group during the second 10-minute subinterval (11-20 minutes) and approximately 25% lower than the control group during the last 10-minute subinterval (51-60 minutes).

A significant (p=0.044) treatment-by-sex interaction was noted when mean total counts were evaluated on PND 61; therefore, the repeated measures analysis of variance was conducted by sex. There were no statistically significant differences in mean total counts for males on PND 61. A significant (p=0.017) treatment-by-time interaction was noted for females when mean total counts were evaluated on PND 61. However, when subsequent pairwise comparisons were conducted, the treatment-by-time interaction was only significant (p=0.002) at 25 ppm. This non-dose-responsive decrease in mean total counts was not considered test substance-related. There was also a significant (p=0.002) treatment effect for females for mean total counts on PND 61; however, subsequent pairwise comparisons were not conducted because of the aforementioned treatment-by-time interaction in accordance with the protocol.

Table 4.11.2.1.Study 3.5 (DAR Table B.6.7.2/1-5): Mean (±S.D.) motor activity data (total activity counts for session)

Table 11. Mean (±S.D.) motor activity data (total activity counts for session)				
Test Day	**Dose (ppm)**			
	0 (N=20)	**25 (N=20)**	**100 (N=20)**	**400 (N=20)**
Males+Females (pooled data)				
PND 13	1585±985.3	1499±111.7 (-5.4)	1688±897.7 (6.5)	2411±2732.8 (52.1)
PND 17	3130±2745.6	2687*±1630.4 (-14.2)	2855*±2561.5 (-8.8)	2339±1550.0 (-25.3)
PND 21	2498±806.5	2198±751.0 (-12.0)	2937±1624.9 (17.6)	2790±1350.6 (11.7)
PND [61]	5559±1475.1	4691±1386.1 (15.6)	5613±1923.6 (1.0)	4723±1403.9 (-15.0)
Males				
PND [61]	5043±1051.1	4306±1215.5 (-14.6)	4527±1376.5 (-10.2)	4499±1104.2 (-10.8)
Females				
PND [61]	6050±1668.8	5076*±1467.6 (-16.1)	6698±1794 (10.7)	4947±1649 (-18.2)

N = number of litters.
Number in brackets (#0=) is percent difference from control.
[*Include units for measurements, as needed.]*
* Statistically different from control, p<0.05
Source: Table 41, pp. 257-269 and Table A41, pp. 1571-1602, Statistics in Appendix J, pp. 2366-2378 in the study report.

Auditory startle response: The auditory startle response habituation paradigm was conducted as a longitudinal assessment with selected pups evaluated on PND 20 and again at sexual maturity (PND 60). Administration of 25, 100, and 400 ppm sulfoxaflor to the maternal animals had no significant effect on auditory startle responsiveness. At PND 20 and 60, the MAX and TMAX values for each of

the 5 blocks of trials evaluated (trials 0-10, 11-20, 21-30, 31-40, and 41-50) were generally similar for the litters in the control and test substance-exposed groups. There was a significant (p=0.026) treatment-by-trial interaction for MAX on PND 60. When subsequent pairwise comparisons were conducted, significant treatment-by-trial interactions were noted at 100 (p=0.016) and 400 (p=0.019) ppm; however, the differences were the result of transient, higher MAX values at these dose levels during the second 10-trial block (trials 11-20) when compared to the control group. This transient increase in MAX values likely represented normal variability in auditory startle response measurements; during the subsequent 10-trial block (trials 21-30), the mean MAX values in the 100 and 400 ppm groups were slightly lower than the control group value. During this interval (trials 21-30) and all other trial blocks of the PND 60 test session (trials 0-10, 31-40, and 41-50), MAX values in the 100 and 400 ppm groups were similar to or slightly lower than the control group values. No other statistically significant differences from the control group were noted when MAX values were analyzed by a repeated measures analysis. No effects were noted in the pattern of the habituation response over the entire 50-trial test session in adult animals.

Table 4.11.2.1.Study 3.6 (DAR Table B.6.7.2/1-6): Mean (±SD) overall (Blocks 1-5) acoustic startle peak amplitude (Newtons) and latency to peak (msec)

Mean (±SD) overall (Blocks 1-5) acoustic startle peak amplitude (Newtons) and latency to peak (msec)[a]					
Dose (ppm)	**Parameter**	**Males**		**Females**	
		PND 20	**PND 60**	**PND 20**	**PND 60**
0	Peak Amp.	1.275±0.385	1.339±0.678	1.186±0.278	1.578±2.087
	Latency	61.02±2.911	45.040±10.390	58.910±4.246	49.180±7.431
25	Peak Amp.	1.385±0.270	1.054±0.566	1.300±0.327	1.126±0.519
	Latency	61.155±2.199	45.970±9.458	60.080±3.250	46.545±8.574
100	Peak Amp.	1.313±0.392	1.430±0.787	1.310±0.300	1.453±0.787
	Latency	61.120±3.780	49.385±12.811	60.720±2.721	50.115±8.930
400	Peak Amp.	1.423±0.390	1.780±1.447	1.422±0.370	1.319±0.662
	Latency	61.430±2.774	42.310±8.211	60.470±3.290	47.845±7.234

Mean and SD overall, calculated by reviewer; n=5.
Source: Table 42, pp. 270-273 and Table A42, pp. 1603-1618.

Biel maze swimming trials: Swimming ability on day 1 of the Biel maze assessment (PND 22 or PND 62) was similar between the control, 25, 100, and 400 ppm groups.

There were no test substance-related effects on the mean numbers of errors committed in Path A (trials 1-4), Path B (trials 5-10), or the repeat of Path A (memory probe; trials 11-12) on PND 22 or 62. A significant (p=0.021) treatment-by-sex interaction was noted when the mean number of errors for Path B (trials 5-10) was evaluated on PND 62; therefore, the repeated measures analysis of variance was conducted by sex. When analyzed by sex, there were no statistically significant differences in the PND 62 mean number of errors committed between the control and test substance-exposed groups for Path B.

There were no biologically meaningful trends for the times to criterion (mean time to locate the submerged platform) during the learning and memory trials between the F_1 males and females in the test substance-exposed groups and the control group beginning on PND 22 and 62.

Table 4.11.2.1.Study 3.7 (DAR Table B.6.7.2/1-7): Biel swimming trials – Male+Female (mean ± S.D.)

Biel swimming trials – Male+Female (mean ± S.D.)					
Test day/parameter		Dose (ppm)			
		0	25	100	400
PND [22] N=20					
Test day 1	Swimming ability (sec)	12.79±2.555	12.61±2.227	12.19±2.914	12.75±3.866
Test day 2 Path A	Time (sec) (first trial)	88.92±35.583	81.63±39.751	89.73±46.021	80.26±37.035
	Errors	16±6.8	15±7.7	17±8.3	15±8.5
	Time (sec) (second trial)	69.91±39.034	80.84±45.610	65.73±35.178	71.96±26.330
	Errors	12±7.5	16±9.3	14±7.9	14±4.9
Test day 3 Path A	Time (sec) (first trial)	71.31±39.335	61.22±29.189	61.25±33.513	63.97±33.002
	Errors	16±9.5	14±8.5	15±10.3	14±8.7
	Time (sec) (second trial)	63.10±40.897	57.83±33.304	55.67±29.876	51.44±37.843
	Errors	14±11.5	13±7.8	12±6.8	10±7.8
Test day 4 Path B	Time (sec) (first trial)	152.43±30.632	148.08±40.822	129.83±50.844	164.79±22.874
	Errors	32±8.9	32±10.9	28±12.1	33±8.1
	Time (sec) (second trial)	117.41±46.492	123.02±44.823	119.33±54.715	126.52±45.456
	Errors	25±11.1	25±8.6	26±13.5	26±10.3
Test day 5 Path B	Time (sec) (first trial)	112.70±48.097	103.65±46.663	110.99±43.428	107.17±50.284
	Errors	23±10.7	21±8.7	24±11.2	21±10.1
	Time (sec) (second trial)	83.06±45.041	95.73±55.043	89.31±41.685	84.38±48.819
	Errors	17±10.9	20±13.9	21±11.3	17±11.1
Test day 7 Recall	Time (sec) (first trial)	80.26±40.777	75.77±33.615	68.99±28.015	68.46±22.948
	Errors	21±10.6	21±10.7	19±7.6	20±6.7
	Time (sec) (second trial)	61.80±30.519	59.53±28.313	44.61±16.893	63.68±38.345
	Errors	15±8.5	15±8.2	11±6.0	16±9.7
PND [62] N=39 (control) and N=40 (25, 100, 400 ppm)					
Test day 1	Swimming ability (sec)	6.02±1.686	6.36±1.689	6.39±2.892	6.23±1.784
Test day 2 Path A	Time (sec) (first trial)	74.54±45.769	10.91±48.185	69.89±45.050	75.00±52.836
	Errors	17±11.4	15±10.9	16±11.6	16±11.7
	Time (sec) (second trial)	46.24±37.270	42.05±37.916	56.05±52.564	59.16±42.955
	Errors	11±9.5	8±7.9	13±11.5	14±10.2
Test day 3 Path A	Time (sec) (first trial)	38.62±31.186	40.47±36.716	50.13±48.495	38.33±37.901
	Errors	10±9.7	10±10.5	14±15.5	10±11.1
	Time (sec) (second trial)	23.06±11.527	26.96±29.753	29.78±33.291	26.90±21.442
	Errors	5±3.5	5±7.7	7±9.4	6±7.2
Test day 4 Path B	Time (sec) (first trial)	139.05±51.854	139.73±56.879	143.61±51.389	139.74±55.813
	Errors	31±12.3	31±13.8	33±13.3	31±12.9
	Time (sec) (second trial)	102.73±64.883	95.20±59.766	101.36±62.772	112.19±
	Errors	20±13.7	20±14.1	22±14.3	22±12.6
Test day 5 Path B	Time (sec) (first trial)	78.28±66.303	75.39±61.291	71.65±55.633	84.84±56.284
	Errors	17±15.9	17±14.6	15±11.7	19±12.0
	Time (sec) (second trial)	61.54±57.181	62.56±55.574	59.28±56.699	55.86±53.030
	Errors	13±13.4	13±12.5	11±10.4	12±13.9
Test day 7 Recall	Time (sec) (first trial)	70.73±48.544	67.42±49.533	85.53±55.901	60.29±41.260
	Errors	20±14.9	20±16.9	26±18.3	17±11.5
	Time (sec) (second trial)	43.90±44.282	46.86±36.058	47.77±40.279	51.81±44.617
	Errors	10±12.2	11±10.3	12±10.9	13±11.8

Path A = forward through maze; Path B = reverse through maze; Time = mean time to escape; Error = all four feet into an incorrect channel.
Source: Tables 43-48, pp.274-295 and Tables A43-A46, pp. 1619-1668 in the study report.

Offspring postmortem results:

Unscheduled Deaths: Male no. 49937-05 in the control group and female no. 49873-10 in the 400 ppm group were found dead on PND 22 and 28, respectively. No remarkable internal findings were noted for either of these animals at necropsy. All other animals survived to the scheduled necropsies.

Animals Euthanized Following PND 22 Learning and Memory (Subset B):

There were no internal findings related to maternal exposure to the test substance noted for Subset B animals euthanized following completion of the learning and memory assessments. Internal findings noted in the test substance-exposed groups consisted of a depressed area on the kidney, swollen spleen, a distended ureter, and clear contents in the uterus. These findings were observed in single animals, in a manner that was not exposure-related, and/or are common findings in laboratory animals. Dilated renal pelves were noted for 3 males in the 400 ppm group. Because this finding was also noted in a single control group female and is a common finding in this species, the slightly increased number of pups with dilated renal pelves in the 400 ppm group males was not considered test substance-related. No other internal findings were noted.

Neuropathology:

PND 21:

Macroscopic Examinations: No test substance-related gross findings were noted in the brain or spinal cord in offspring selected for brain weights on PND 21. In the 25 ppm group, 1 male had dark red material attached to the brain and 1 female had a depressed area on the brain; these findings were not observed at higher exposure levels.

Brain Weights/Brain Measurements: No test substance-related effects on mean brain weights or measurements were noted at any exposure level on PND 21. Mean brain width (combined-sex) in the 100 ppm group (14.4 mm) was significantly ($p=0.002$) higher than the control group value (14.2 mm); however, in the absence of a dose response, the difference was not considered test substance-related. Other differences between the control and test substance-exposed groups were slight and not statistically significant.

Table 4.11.2.1.Study 3.8 (DAR Table B.6.7.2/1-8): Mean (±SD) brain weight data

Mean (±SD) brain weight data				
Parameter	**Dose (ppm)**			
	0	**25**	**100**	**400**
Males				
Day 21 [N=20]				
Terminal body weight (g)	44.81±4.16	47.30±4.69	46.15±5.23	44.21±6.40
Brain weight (g)	1.5337±0.08767	1.5862±0.07528	1.5458±0.07739	1.5415±0.07611
Brain-to-body weight ratio	3.42%	3.35%	3.35%	3.49%
Termination [N=19-20]				
Terminal body weight (g)	421.326±40.7786	426.75±36.8980	425.50±44.047	394.60±43.893
Brain weight (g)	2.1468±0.07311	2.1270±0.07540	2.0765±0.12149	2.0355*±0.14848
Brain-to-body weight ratio	0.510%	0.498%	0.488%	0.516%
Females				
Day 21 [N=20]				
Terminal body weight (g)	43.03±4.72	44.14±4.68	44.23±4.47	43.414±5.49
Brain weight (g)	1.4980±0.08739	1.5165±0.08105	1.5024±0.07790	1.4855±0.06444
Brain-to-body weight ratio	3.48%	3.44%	3.40%	3.42%
Termination [N=20]				
Terminal body weight (g)	253.15±20.5562	251.75±29.4563	253.85±21.777	249.70±25.787
Brain weight (g)	1.965±0.08630	2.0050±0.09417	1.9620±0.10144	1.9295±0.10531
Brain-to-body weight ratio	0.776%	0.796%	0.773%	0.773%

N = 40
* Statistically different from control, p<0.05
** Statistically different from control, p<0.01
Source: Tables 54 and 58, pp.302-304, and TablesA52 and A55, pp. 1990-1997, 2175-2182 in the study report.

Microscopic Examinations: There were no test substance-related histologic changes in the brain of males and females at any exposure level on PND 21. All histologic changes were considered to be incidental findings or related to some aspect of experimental manipulation other than administration of the test substance. There were no test substance-related alterations in the prevalence, severity, or histologic character of those incidental tissue alterations.

Table 4.11.2.1.Study 3.9 (DAR Table B.6.7.2/1-9): Histopathology findings

Histopathology findings					
Parameter		**Dose (ppm)**			
		0 (N=10)	**25 (N=10)**	**100 (N=10)**	**400 (N=10)**
		Males			
		Day 21 [N=10]			
Basal Ganglia – ectopic tissue	minimal	1	NA	NA	0
		Day Termination [N=10]			
Lum spin nerve -degeneration, axonal	minimal	1	NA	NA	1
Lumbar dor. fib. -degeneration, axonal	minimal	0	NA	NA	2
Peroneal nerve -degeneration, axonal	minimal	1	NA	NA	3
Sciatic nerve	minimal	3	NA	NA	4
unremarkable	mild	1	NA	NA	0
Tibial nerve -degeneration, axonal	minimal	1	NA	NA	2
Trigeminal nerve -degeneration, axonal	minimal	0	NA	NA	1
		Females			
		Day 21 [N=10]			
None found					
		Termination [N=10]			
Lum spin nerve -degeneration, axonal	minimal	1	NA	NA	1
Lumbar dor. fib. -degeneration, axonal	minimal	1	NA	NA	1
Lumbar vent. fib-degeneration, axonal	minimal	1	NA	NA	0
Sciatic nerve degeneration, axonal	minimal	5	NA	NA	1

N = 10
NA = not applicable, none examined from this dose level.
* Statistically different from control, p<0.05
** Statistically different from control, p<0.01
Source: Tables 55 and 59, pp. 305-308, 321-332 and Tables A53 and A56, pp. 1998-2015, 2183-2198 in the study report.

There were no differences between the control and 400 ppm rats in any of the mean brain morphometry measurements on PND 21.

PND 72:

Macroscopic Examinations: no test substance-related gross findings were noted in the brain or spinal cord in offspring selected for brain weights on PND 72,. The only macroscopic finding noted in the test substance-exposed groups was dark red material attached to the brain for 2 males in the 25 ppm group; this finding was also noted for 1 male in the control group, and was not observed at higher exposure levels. One male in the control group also had a dark red area on the brain and a small brain (olfactory bulb).

Brain Weights/Brain Measurements: No test substance-related effects on mean brain weights or measurements were noted at any exposure level on PND 72. A significant (p<0.001) treatment-by-sex interaction was noted when mean brain weights and measurements were evaluated on PND 72; therefore, the multivariate analysis of variance was conducted by sex. When analyzed for each sex separately, mean absolute brain weight (p=0.002) and brain length (p<0.001) for the 400 ppm group

males were significantly lower (5% and 4%, respectively) than the control group. However, in the 400 ppm group females, the mean absolute brain length was significantly ($p<0.001$) higher (4%) than the control group.

The relationship with treatment of the apparent decreased brain length and weight in males is equivocal. The differences in brain weight and length were small (4-5%) and likely within the natural variance. There was no pattern of alteration in gross or microscopic brain structures and no differences in brain morphometric values in either sex. There were no treatment-related effects on mean brain weights or measurements noted at any exposure level on PND 21 as treatment of the animals was discontinued at this time point (21days). Decrease in male brain weight/length on day PND 72 is not likely to be treatment-related when there were no alterations in these measurements on PND 21.

In CD rats, the strain used for the study, brain and body weights continue to rise during the life span of the animals. Furthermore published studies show variability in brain weights across similarly aged animals, particularly in the first 100 days of life.

Microscopic Examinations: There were no test substance-related histologic changes in the brain of males and females at any exposure level on PND 72. There were instances of axonal degeneration in the peripheral nerves, particularly the sciatic nerve, and sometimes in the spinal nerve roots. This axonal degeneration was of minimal severity, typically with only a single 'digestion chamber', and consistent with incidental alterations. Minimal axonal degeneration in the peripheral nerves and spinal nerve roots is a common background lesion. In addition, the relative incidence of minimal axonal degeneration in the lumbar dorsal fibers and peroneal nerves for males in the 400 ppm group (20% and 30%, respectively) was similar to that noted in the WIL historical control data (17.8% and 36%, respectively).

There were no differences between the control and 400 ppm rats in any of the mean brain morphometry measurements on PND 21.

Conclusions

There were no test substance-related effects on maternal parameters in this study. Therefore, the no-observed-adverse-effect-level (NOAEL) for systemic toxicity and maternal reproductive toxicity of sulfoxaflor when administered orally in the diet was 400 ppm (equivalent to 28.8 mg/kg/day).

Offspring LOAEL was based on the reduction in postnatal survival, decreased pup body weights, delayed righting reflex at 400 ppm. The apparent decrease in brain weight in males, and altered brain length in males and females (400 ppm at 72 days only), was not considered related to treatment. The NOAEL for neonatal toxicity was 100 ppm (equivalent to 7.4 mg/kg/day

4.11.2.2 Human information

No data available.

4.11.3 Other relevant information

In addition to standard regulatory studies, comprising a two-generation reproduction study in rats and a developmental study in rats and rabbits, a series of studies was conducted to understand the mode of action for two effects seen in rats – 1) foetal abnormalities (primarily forelimb flexure and bent clavicle plus hindlimb rotation, and convoluted/hydroureter) and 2) neonatal pup loss at birth. Apart from a slight delay in balano-preputial separation (BPS) in high dose level male CD rats these

were the only treatment-related reproduction effects of sulfoxaflor.

The developmental mode of action (MoA) program and related tests were based on a hypothesis that both effects had a single MoA associated with sulfoxaflor's agonism to the foetal rat muscle nicotinic acetylcholine receptor (nAChR). A series of investigatory studies in rats and rabbits and *in vitro* studies using recombinant rat and human nAChRs investigated the possibility that the developmental target for sulfoxaflor is the foetal rat muscle nicotinic acetylcholine receptor. Prolonged activity (agonism) at this receptor in rats causes striated muscle contracture and reduced muscle responsiveness, considered responsible for the foetal abnormalities and neonatal death in the rat. All morphological effects in foetal rats (primarily forelimb flexure and bent clavicle plus hindlimb rotation, and convoluted/hydro ureter) were shown to be reversible after birth. Therefore, these were shown to be pharmacological effects mediated *via in utero* exposure from the mother at the end of gestation. Sulfoxaflor was also shown not to be an agonist to the corresponding human receptors.

Only the executive summaries are provided below. The study details are provided in the relevant Annex.

4.11.3.1 Mechanistic investigations

Study 1: Rat cross-fostering study (DAR B.6.6.12.)

Report:	XDE-208: A Dietary Reproductive Toxicity Cross-Fostering Study in Crl:CD(SD) Rats
Author:	R. J. Rasoulpour, Ph., Zablotny, C.L. (2010d)
Date of Report:	01 July, 2010
Report Identity:	Study ID: 081122
Testing Facility:	Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan, 48674.
GLP	Yes
Test Substance:	XDE-208 (95.6% (wt/wt); as two diastereomers in 48.4/47.4% ratio.
Batch:	E2162-34 TSN003725-0001
Guidelines:	Non-guideline
Deviations:	Not applicable
Acceptable:	Yes

The purpose of this study was to determine whether the previously observed decreased survival of pups born to sulfoxaflor-treated dams resulted from *in utero* and/or lactational exposure. As part of this study, effects on general toxicity, toxicokinetic analysis of blood and milk, reproductive function and prenatal/early neonatal growth and survival were assessed.
Groups of female Crl:CD(SD) rats were fed diets supplying 0 (control) or 1000 ppm sulfoxaflor for two weeks prior to mating through weaning on lactation day (LD) 21. As the control and treated females mated, they were subdivided into Foster dams and Donor dams. Cesarean-section was performed on gestation day (GD) 21 Donor dams, at which time, one or more batches of two of their offspring/sex were immediately cross-fostered to a Foster dam(s) that had their own litter removed that day (i.e., on LD 0). After cross-fostering was complete, each control and sulfoxaflor-treated Foster dam had mixed litters comprised of two pups/sex that originated from control Donor dams (five litters) and two pups/sex that originated from sulfoxaflor-treated Donor dams (eight litters). This design controlled for litter of origin effects, and enabled comparison of the survival of pups exposed to sulfoxaflor during gestation alone or during lactation alone with unexposed control pups and pups exposed during both gestation and

lactation.
Dams given 1000 ppm sulfoxaflor had treatment-related effects on body weight, body weight gain, and feed consumption consistent with effects seen at this dose level in the previous reproduction/developmental toxicity screening study. Time weighted average doses for treated animals were 81.2, 74.5, and 59.5 mg/kg/day in the pre-mating, gestation, and lactation periods, respectively. These corresponded to maternal sulfoxaflor blood concentrations of 23.0-29.3 µg/g plasma on GD 21 and 19.6-25.0 µg/g plasma on LD 0. The average measured plasma concentration of sulfoxaflor of male/female pups on GD 21 and LD 0 from these dams was 24.8/24.8 and 25.3/25.9 µg sulfoxaflor/g plasma, respectively. Thus, foetal and pup plasma levels of sulfoxaflor were very similar to one another, and very similar to dam plasma levels. The measured milk concentration from the same dams on LD 0 were approximately half the corresponding plasma levels and ranged from 12.3-14.0 µg sulfoxaflor/g milk (mean = 13.3 µg/g)
All offspring from dams exposed to 1000 ppm sulfoxaflor prior to birth died by postnatal day (PND) 4, irrespective of whether they were cross-fostered to control- or treated-foster dams (see results table below). Consistent with reduced viability, some offspring were cold to the touch, had bluish skin, autolysed and cannibalised, and stomach void of milk. Conversely, there was no effect on neonatal survival for pups exposed to sulfoxaflor only after birth. Furthermore, PND 1 pup body weights were significantly decreased in prenatally exposed offspring. In conclusion, these data demonstrate that the effect of sulfoxaflor on pup survival was due to *in utero*, not lactational, exposure.

Table 4.11.3.1.Study 1.1 (DAR Table Cross Foster or Treated Foster Dams Results)

Foster Dams	Donor Pups	Hypotheses for Pup Survival	Outcome
Control	Control *in utero*	**No effect expected** (negative control)	No effect
	Treated *in utero*	If pups die, effect comes from treated **pups** (i.e., ***in utero* effect**)	All pups died by PND4
XDE-208 1000 ppm	Control *in utero*	If pups die, effect comes from treated **dams** (i.e., **lactational effect**)	No effect
	Treated *in utero*	**Pup death expected** (positive control)	All pups died by PND4

This study non-guideline study is acceptable.

Conclusions
Dams given 1000 ppm sulfoxaflor had treatment-related effects on body weight, body weight gain, and feed consumption consistent with effects seen at this dose level in the previous reproduction/developmental toxicity screening study. Time weighted average doses for treated animals were 81.2, 74.5, and 59.5 mg/kg/day in the pre-mating, gestation, and lactation periods, respectively. These corresponded to maternal sulfoxaflor blood concentrations of 23.0-29.3 µg/g plasma on GD 21 and 19.6-25.0 µg/g plasma on LD 0. The average measured plasma concentration of sulfoxaflor of male/female pups on GD 21 and LD 0 from these dams was 24.8/24.8 and 25.3/25.9 µg sulfoxaflor/g plasma, respectively. Thus, foetal and pup plasma levels of sulfoxaflor were very similar to one another, and very similar to dam plasma levels. The measured milk concentration from the same dams on LD 0 were approximately half the corresponding plasma levels and ranged from 12.3-14.0 µg sulfoxaflor/g milk (mean = 13.3 µg/g)
All offspring from dams exposed to 1000 ppm sulfoxaflor prior to birth died by postnatal day (PND) 4, irrespective of whether they were cross-fostered to control- or treated-foster dams (see

results table below). Consistent with reduced viability, some offspring were cold to the touch, had bluish skin, autolysed and cannibalised, and stomach void of milk. Conversely, there was no effect on neonatal survival for pups exposed to sulfoxaflor only after birth. Furthermore, PND 1 pup body weights were significantly decreased in prenatally exposed offspring. In conclusion, these data demonstrate that the effect of sulfoxaflor on pup survival was due to *in utero*, not lactational, exposure.

Study 2: Rabbit neonatal survival study (DAR B.6.6.12.2)

Report:	A Study of the Effect of XDE-208 on Neonatal Survival in New Zealand White Rabbits
Author:	Kuhl, A.J.
Date of Report:	04 August, 2009
Report Identity:	Study ID: WIL-410011
Testing Facility:	WIL Research Laboratories, LLC, Ashland, OH, 2009.
GLP	Yes
Test Substance:	XDE-208 (95.6% (wt/wt); as two diastereomers in 48.4/47.4% ratio.
Batch:	E2162-34
Guidelines:	Non-guideline
Deviations:	Not applicable
Acceptable:	Yes

Sulfoxaflor, was offered on a continuous basis in the diet (with 0.5% apple flavoring) to a group of 12 litter-experienced, time mated female New Zealand White [Hra:(NZW)SPF] rabbits from gestation day (GD) 7 through the initiation of parturition (25-26 consecutive days). The target test substance concentration of 750 ppm was achieved (101.5% of target concentration), and reflected a maximum tolerated exposure based on previous studies in this species. Actual test material intake in the 750 ppm group was 29 mg/kg/day during GD 7-28. A concurrent control group of 12 time-mated females received the apple-flavored control diet on a comparable regimen. All diets were formulated according to the specifications for Purina Mills International (PMI) Certified Rabbit LabDiet® 5325 and were provided at 150 g/day ± 5 g/day during the exposure period (GD 7 through initiation of parturition) and at 200 g/day ± 5 g/day during lactation days (LD) 1-4; the control diet was offered to both groups after parturition. The F0 females were approximately 9-13 months of age at the initiation of test substance exposure. All animals were observed twice daily for mortality and moribundity. Clinical observations, body weights, and food consumption were recorded at appropriate intervals. All F0 females were allowed to deliver and rear their offspring to LD 4. All F0 females were necropsied within 24 hours of total litter loss, on LD 4, or on post mating day 37. All surviving F1 offspring received a detailed physical examination on postnatal day (PND) 4 and were then discarded.

With the exception of 1 F0 female in the control and 750 ppm groups euthanized on LD 3 due to total litter loss, all females survived to the scheduled necropsies. No test substance-related maternal macroscopic findings were noted.

Lower mean body weight gains (24.2%) and food consumption (7.3%) were noted in the 750 ppm group during the gestation exposure period compared to the control group. Corresponding incidences of decreased defecation were noted for 3 females in this group. Although mean body weights remained within 2.9% of control group values throughout gestation, the reductions in mean body weight gains and food consumption were attributed to test substance exposure. Mean body weights, body weight gains, and food consumption in the 750 ppm group were similar to the control group during LD 1-4.

No test substance-related effects were observed on the mean number of offspring born, offspring survival, or the general physical condition of the offspring.

Based on these results, an exposure level of 750 ppm, equivalent to 29 mg/kg/day, was considered to be the no observed effect level (NOEL) for neonatal survival when sulfoxaflor was offered continuously in the diet from GD 7 through the initiation of parturition to pregnant New Zealand White rabbits. In contrast to the rat, sulfoxaflor was not developmentally toxic in the rabbit, despite the achievement of similar maternal and foetal systemic concentrations of sulfoxaflor in both species.

Conclusion

Lower mean maternal body weight gains and food consumption and corresponding clinical findings of decreased defecation were noted in the 750 ppm group which were attributed to test substance exposure, but are not considered toxicologically significant. No test substance related effects were noted on postnatal survival or the general condition of the F1 offspring. 750 ppm (29 mg/kg/day) was considered an NOAEL for both maternal and offspring effects.

Study 3: In-vitro mode of action study in the rat, rabbit, and human (DAR B6.6.12.3).

Report:	XDE-208: Characterization of the agonist effects of XDE-208 on mammalian muscle nicotinic acetylcholine receptors.
Author:	Millar, N.
Date of Report:	7th June, 2010
Report Identity:	Study ID: UCL nAChR
Testing Facility:	Research Department of Neuroscience, Physiology & Pharmacology, University College London (London UK)
GLP	Signed and dated GLP (non-compliance) and (No) Data Confidentiality statements were provided. A Quality Assurance statement was not provided.
Test Substance:	XDE-208 (95.6% (wt/wt); as two diastereomers in 48.4/47.4% ratio. X11719474, a soil metabolite of XDE-208.
Batch:	E2162-34 XS9-37307-78
Guidelines:	Non-guideline
Deviations:	Not applicable
Acceptable:	Yes

Sulfoxaflor is a compound with insecticidal activity that acts as an agonist of insect nicotinic acetylcholine receptors (nAChRs). The aim of the work described in this report was to examine the influence of sulfoxaflor on mammalian muscle nAChRs. Competition radioligand binding was used to examine the ability of sulfoxaflor to bind to nAChRs from three mammalian species (human, rabbit and rat). In addition, two-electrode voltage-clamp recording was used (with human and rat nAChRs) to examine whether binding of sulfoxaflor resulted in functional activation of muscle nAChRs. Radioligand binding experiments demonstrated that sulfoxaflor binds to human, rabbit and rat foetal muscle nAChRs. Electrophysiological studies revealed that sulfoxaflor is a partial agonist of the rat foetal muscle nAChR. In contrast, sulfoxaflor has no detectable agonist activity on the human foetal muscle nAChR or on the adult muscle nAChR (from either human or rat). In contrast to the clear agonist activity of sulfoxaflor on the rat foetal muscle nAChR, no agonist activity was observed with X11719474, a soil metabolite of sulfoxaflor. This non-guideline study *in-vitro* mode of action study was considered acceptable.

Conclusions:

The work described in the present study demonstrates that sulfoxaflor is an agonist of the rat foetal

muscle nAChR (which contains the rat γ subunit). In contrast, sulfoxaflor has no agonist activity on the equivalent human nAChR (containing the human γ subunit) or on the rat or human adult muscle nAChR (containing the rat or human ε subunit). From these findings, it seems reasonable to conclude that the selective agonist activity of sulfoxaflor is due to differences in the amino acid sequence of the rat γ subunits compared with that of the human γ subunit (and also with the rat and human ε subunit).

Study 4: Critical window Phase 1

Report:	XDE 208: Investigation of the critical window of exposure for fetal abnormalities and neonatal survival effects in Crl:CD(SD) rats.
Author:	Rasoulpour, R. and C. Zablotny
Date of Report:	**2**5th June, 2010
Report Identity:	Study ID: 091022
Testing Facility:	Toxicology & Environmental Research and Consulting, The Dow Chemical Company (Michigan).
GLP	Signed and dated GLP (non-compliance), Quality Assurance, and (No) Data Confidentiality statements were provided. .
Test Substance:	XDE-208 (95.6% (wt/wt); as two diastereomers in 48.4/47.4% ratio.
Batch:	E2162-34
Guidelines:	Non-guideline
Deviations:	Not applicable
Acceptable:	Yes

Exposure to 1000 ppm sulfoxaflor throughout gestation (gestation days (GD) 6-21) has been previously shown to cause foetal limb contractures (forelimb flexure and hindlimb rotation) and reduced neonatal survival. It was hypothesised that these effects might result from agonism of sulfoxaflor at the foetal muscle nicotinic acetylcholine receptor (nAChR) based on information available at the time, which indicated 1) this is consistent with the molecule's insecticidal mode-of-action, 2) a soil metabolite of sulfoxaflor (X11719474), which does not bind to the insect nAChR, did not induce limb contractures or reduced neonatal survival even at very high dose levels, and 3) this muscle receptor subtype is highly expressed during late gestation in the distal limbs muscles and diaphragm, with impairment of diaphragmatic maintenance of respiration at birth implicated in neonatal death from sulfoxaflor exposure.

This was the first of two studies conducted to determine the critical window of susceptibility, and to test the hypothesis that late gestational exposure to sulfoxaflor induces foetal abnormalities and reduced neonatal survival *via* its pharmacological action on the foetal muscle nicotinic acetylcholine receptor (nAChR). This receptor develops functional expression between GD 16 and 17 in the rat, resulting in synchronised foetal limb movements and diaphragmatic responsiveness important for the transition to extrauterine respiration.

In this study, groups of 12 female Crl:CD(SD) rats were administered control diet (Group 1), or diets containing 1000 ppm sulfoxaflor fed from GD 6-16 (Group 2) to cover all of embryogenesis up to, but not including, the start of early foetal movements, or 1000 ppm sulfoxaflor fed from GD 16-birth (Group 3) to cover development of the muscle nAChR and its role in development of synchronised foetal limb movements up to onset of parturition. In the offspring, effects on litter size, survival, body weight and the presence of gross external morphological alterations, with particular focus on limb abnormalities (e.g., forelimb flexure and hindlimb rotation), were carefully assessed. In addition, a subset of animals was examined for the presence of convoluted ureters and bent clavicles as these effects had also been seen in the sulfoxaflor rat developmental toxicity study at 1000 ppm.

Offspring from animals given 1000 ppm sulfoxaflor from GD 6-16 (Group 2) were completely normal and did not display previously described foetal abnormalities or reduced neonatal survival. In contrast, offspring given 1000 ppm sulfoxaflor from GD 16-birth (Group 3) had the same gross effects of limb contractures and reduced neonatal survival seen in the previous studies that had treatment with 1000 ppm sulfoxaflor throughout gestation. This demonstrates that the critical window of susceptibility for both of these effects falls within GD 16-birth.

In addition, daily examination of Group 3 offspring born with limb abnormalities indicated that these were fully reversible shortly after withdrawal of maternal dietary exposure to sulfoxaflor. In some cases, full reversal of the limb abnormalities was evident the day after birth and occurred for all affected animals that survived to postnatal day (PND) 4; reversal also occurred in some animals that subsequently died before PND 4. Likewise, the visceral and skeletal findings of abnormal ureter and bent clavicle, the latter of which had a high incidence (30.1% of fetuses), in the definitive developmental toxicity study were not present in this study at necropsy on PND 4 despite similar blood concentrations and limb abnormality indices between these two studies.

In summary, this study demonstrated that the critical period of developmental susceptibility to sulfoxaflor-induced foetal abnormalities and reduced neonatal survival is between GD 16-birth, and that all of the foetal abnormalities are rapidly reversible after birth. These results support the hypothesis that late gestational exposure to sulfoxaflor induces foetal abnormalities and neonatal death *via* its pharmacological action on the foetal muscle nicotinic acetylcholine receptor (nAChR), which develops functional expression during this stage of gestation. This non-guideline study is acceptable.

Conclusions

The critical window of susceptibility in rats for the foetal abnormalities of limb contractures and reduced neonatal survival resulting from maternal exposure to 1000 ppm sulfoxaflor *via* the diet falls within the exposure period of GD16-birth. These abnormalities are reversible upon birth upon withdrawal of maternal dietary exposure. These results support the hypothesis that late gestational exposure to sulfoxaflor induces foetal abnormalities and neonatal death *via* its pharmacological action on the foetal muscle nicotinic acetylcholine receptor (nAChR), which develops functional expression during this stage of gestation.

Study 5: Critical window Phase 2

Report:	XDE 208: Investigation of the critical window of exposure for fetal abnormalities and neonatal survival effects in Crl:CD(SD) rats (Phase 2).
Author:	Rasoulpour, R. and C. Zablotny
Date of Report:	**2**4 June, 2010
Report Identity:	Study ID: 091049
Testing Facility:	Toxicology & Environmental Research and Consulting, The Dow Chemical Company (Michigan).
GLP	Signed and dated GLP (non-compliance), Quality Assurance, and (No) Data Confidentiality statements were provided. .
Test Substance:	XDE-208 (95.6% (wt/wt); as two diastereomers in 48.4/47.4% ratio.
Batch:	E2162-34
Guidelines:	Non-guideline
Deviations:	Not applicable

Acceptable: Yes

This was the second of two studies conducted to determine the critical window of susceptibility, and to test the hypothesis that late gestational exposure to sulfoxaflor induces foetal abnormalities and reduced neonatal survival *via* its pharmacological action on the foetal muscle nicotinic acetylcholine receptor (nAChR). This receptor develops functional expression between GD 16 and 17 in the rat, resulting in synchronised foetal limb movements and diaphragmatic responsiveness important for the transition to extrauterine respiration.

The purpose of this study was to further refine the critical window of sulfoxaflor exposure that is sufficient to cause foetal abnormalities and reduce neonatal survival. This study divided the GD 16-birth exposure window - shown in the first study to be the exposure period responsible for both effects - into three 48-hour exposure windows starting on the morning of GD 16, 18, or 20. Groups of 10 female Crl:CD(SD) rats were administered control diet (Group 1), or diets containing 1000 ppm sulfoxaflor (the high dose level from the developmental toxicity study) fed from GD 16-18 (Group 2), GD 18-20 (Group 3), or GD 20-22 (Group 4). In the offspring, effects on litter size, survival, body weight and the presence of gross external morphological alterations, with particular focus on limb abnormalities (e.g., forelimb flexure and hindlimb rotation), were carefully assessed. In addition, a subset of animals was examined for the presence of convoluted ureters and bent clavicles as these effects had also been seen in the sulfoxaflor rat developmental toxicity study at 1000 ppm

Offspring from animals given 1000 ppm sulfoxaflor for 48 hours starting on the morning of GD 16 or 18 (Group 2 and 3) were similar to controls and did not display previously described foetal abnormalities or reduced neonatal survival. In contrast, offspring given 1000 ppm sulfoxaflor for 48 hours starting on the morning of GD 20 (Group 4) had foetal limb abnormalities (forelimb flexure and hindlimb rotation) as well as reduced neonatal survival, demonstrating that exposure shortly before birth (GD 21 or 22) is sufficient to induce developmental toxicity.

In addition, daily examination of surviving Group 4 offspring born with limb abnormalities indicated that these were fully reversible in surviving offspring shortly after withdrawal of maternal dietary exposure to sulfoxaflor. In some cases, full reversal of the limb abnormalities was evident the day after birth and occurred for all affected animals that survived to postnatal day (PND) 4; reversal also occurred in some animals that subsequently died before PND 4. Likewise, the visceral and skeletal findings of abnormal ureter and bent clavicle, the latter of which had a high incidence (30.1% of foetuses), in the definitive developmental toxicity study were not present in this study at necropsy on PND 4.

In summary, this study demonstrated that the critical period of developmental susceptibility to sulfoxaflor-induced foetal abnormalities and reduced neonatal survival effects occurs shortly before birth, and that the foetal abnormalities are rapidly reversible after birth. These results support the hypothesis that late gestational exposure to sulfoxaflor induces foetal abnormalities and neonatal death *via* its pharmacological action on the foetal muscle nicotinic acetylcholine receptor (nAChR), which develops functional expression during this stage of gestation. This study non-guideline study is considered acceptable.

Conclusions

Offspring from animals given 1000 ppm sulfoxaflor for 48 hours starting on the morning of GD 16 or 18 (Group 2 and 3) were similar to controls and did not display previously described foetal abnormalities or reduced neonatal survival. In contrast, offspring given 1000 ppm sulfoxaflor for 48 hours starting on the morning of GD 20 (Group 4) had foetal limb abnormalities (forelimb flexure and hindlimb rotation) as well as reduced neonatal survival, demonstrating that exposure shortly before birth (GD 21 or 22) is sufficient to induce developmental toxicity.

This study demonstrated that the critical period of developmental susceptibility to sulfoxaflor-induced foetal abnormalities and reduced neonatal survival effects occurs shortly before birth (GD 21 or 22), and that the foetal abnormalities are rapidly reversible after birth. These results support the hypothesis that late gestational exposure to sulfoxaflor induces foetal abnormalities and neonatal death *via* its pharmacological action on the foetal muscle nicotinic acetylcholine receptor (nAChR), which develops functional expression during this stage of gestation

Study 6: Diaphragm contracture.

Report:	Observations on the effects of XDE-208 on the phrenic nerve-hemidiaphragm preparation from new-born rat.
Author:	Alasdair J. Gibb, Ph.D. (2010).
Date of Report:	30 June, 2010
Report Identity:	UCL Diaphragm (30 June 2010).
Testing Facility:	Research Department of Neuroscience, Physiology & Pharmacology, University College London, Gower Street, London, WC1E 6BT, United Kingdom.
GLP	Signed and dated Data Confidentiality statements were not provided. .
Test Substance:	XDE-208 (95.6% (wt/wt); as two diastereomers in 50/49.5% ratio.
Batch:	E2162-34
Guidelines:	Non-guideline
Deviations:	Not applicable
Acceptable:	Yes

Sulfoxaflor, a compound targeted to the insect nicotinic acetylcholine receptor (nAChR) has been shown to cause foetal limb contractions and reduced neonatal survival in rats following dietary exposure during gestation. It is hypothesised that these effects result from activation of the foetal muscle-type nAChR bysulfoxaflor, thereby causing sustained muscle contracture in the foetus and inhibition of nerve-evoked contraction of the diaphragm that would cause impaired respiration after birth resulting in the previously observed reductions in neonatal survival. In support of this hypothesis, sulfoxaflor has been demonstrated to be an agonist at rat, but not human, embryonic ((α1)2β1δγ)) nAChR, while being without agonist activity at mature ((α1)2β1δε)) muscle–type nAChRs (rat or human). The aim of the work described in this report was to make a qualitative investigation of the action of sulfoxaflor on isolated phrenic nerve-hemidiaphragm preparations from new-born rats. Sulfoxaflor consistently (n=5) produced a reversible, concentration-dependent contracture of the diaphragm that was blocked by the selective muscle-type nAChR antagonist, tubocurarine (10 µM) showing that the contracture induced by sulfoxaflor is mediated *via* nAChR activation, rather than *via* a post-receptor mechanism. Furthermore, prolonged application of sulfoxaflor caused a sustained muscle contracture. Muscle twitches in response to phrenic nerve stimulation were not affected at low sulfoxaflor concentration (100 µM) but were reduced at high concentration (1 mM) demonstrating that sulfoxaflor can cause inhibition of nerve-evoked contraction of the diaphragm during sustained contracture, consistent with the observed impairment of respiration in the neonatal rat. The results of these experiments demonstrate that sulfoxaflor caused a contracture of the new-born rat diaphragm by acting on the nAChR. Prolonged application caused a sustained muscle contracture and a contracture-associated inhibition of the phrenic nerve-evoked muscle twitch, which is considered analogous to the situation *in vivo* which resulted in foetal limb contractions (sustained muscle contractions) and compromised respiration at birth (contracture-associated inhibition of the muscle twitch). Therefore, the results described in this report are entirely consistent with, and add additional support to, the hypothesis that sulfoxaflor causes neonatal death (and foetal abnormalities) *via* activation of the foetal muscle-type nAChR.

Conclusions

The results of these qualitative experiments demonstrate that sulfoxaflor causes a concentration-dependent contracture of the new-born rat diaphragm via activation of muscle-type nAChRs. Prolonged application of sulfoxaflor caused a sustained muscle contracture and contracture-associated decrease in muscle twitch that is considered analogous to the situation in vivo that resulted in poor survival after birth. Thus the results described herein are entirely consistent with and add additional support to the hypothesis that sulfoxaflor causes neonatal death (and foetal abnormalities) via activation of the foetal muscle nAChR.

Study 7: Foetal Lung contracture

Report:	Histopathological Evaluation Of Fetal Lung Samples From The Developmental Toxicity Study In Crl:Cd(Sd) Rats.
Author:	J. Thomas, Ph.D. and V. A. Marshall, B.S. (2010).
Date of Report:	18 June, 2010
Report Identity:	Study ID: 100124
Testing Facility:	Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan, 48674
GLP	The study is not GLP compliant. However, all experiments were done according to GLP standards.
Test Substance:	XDE-208 (95.6% (wt/wt); as two diastereomers in 50/49.5% ratio.
Batch:	E2162-34
Guidelines:	Non-guideline
Deviations:	Not applicable
Acceptable:	Yes

Dietary administration of 1000 ppm sulfoxaflor to Crl:CD(SD) rats during gestation has been previously shown to cause neonatal pup death. In order to determine if morphological alterations (e.g., increased collagen deposition) in any region of the lungs were responsible for pup death, one foetus/sex from five control and four 1000 ppm litters (18 samples total) from the definitive developmental toxicity study were collected and preserved in neutral, phosphate buffered 10% formalin. Sections from these preserved tissues were processed such that each slide contained sections of the trachea, bronchi, bronchioles, and alveoli. Slides were stained with haematoxylin and eosin and evaluated for histopathological changes. Tissues were archived with the developmental toxicity study.

To detect any morphological abnormalities, including increased collagen deposition in the pulmonary tract, of rat foetuses exposed *in utero* to the high-dose of 1000 ppm sulfoxaflor which may have been contributory to treatment-related increase in neonatal pup mortality.

Two formalin fixed foetuses (one male and one female) per dam from the control group and from dams fed 1000 ppm sulfoxaflor were randomly selected. Five control dams and four dams given 1000 ppm were selected, totaling ten control foetuses and eight sulfoxaflor exposed foetuses. The trachea and the lungs of these selected foetuses were routinely processed for histology, sections cut at 5-6 microns thick, stained with haematoxylin and eosin, and examined by a veterinary pathologist.

There were no sulfoxaflor induced lesions in the trachea, bronchi, bronchioles and alveoli in any of the treated foetuses examined. There were no treatment-related increases in collagen deposition around the airways or alveolar walls or any other changes. All observations were considered within normal limits. Therefore, histopathologic examination of the trachea and lungs of selected foetuses from dams given 1000 ppm sulfoxaflor from GD 6-21 did not reveal any morphologic abnormalities in the trachea or within the lungs that could have contributed to 1000 ppm sulfoxaflor induced neonatal mortality in rat pups.

Conclusion

Histopathologic examination of the trachea and lungs of selected foetuses from dams given 1000 ppm sulfoxaflor from GD 6-21 did not reveal any morphologic abnormalities in the trachea or within the lungs that could have contributed to 1000 ppm sulfoxaflor induced neonatal mortality in rat pups

Study 8: Human Relevance Framework

The following section summarises the notifiers evaluation of the reproductive and developmental data including the MoA studies according to the Bradford-Hill criteria and the subsequent application of the Human Relevence Framework.

Report:	Sulfoxaflor: Mode of action evaluation and human relevance framework analysis for Sulfoxaflor-induced foetal abnormalities and neonatal death in rats.
Author:	R. G. Ellis-Hutchings, Ph.D., R. J. Rasoulpour, Ph.D., C. Terry, Ph.D., B. B. Gollapudi, Ph.D., and R. Billington, M.Sc., DABT, DRCPath
Date of Report:	**7th** December 2010
Report Identity:	Study ID: 100290
Testing Facility:	Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan, 48674
Acceptable:	Yes

Sulfoxaflor, an insecticide that operates *via* the insect nicotinic acetylcholine receptor (nAChR), causes foetal abnormalities (primarily limb contractures) and death in neonatal rats, but not rabbits, following high dose dietary exposure during gestation in regulatory guideline studies. It has been proposed that these effects have a single mode of action (MoA) mediated *via* the rat foetal-type muscle nAChR through the following key events: (1) binding to the receptor, (2) agonism (activation) at the receptor, causing (3) sustained muscle contracture in the near-term foetus and neonatal offspring. This sustained muscle contracture results in limb contractures, bent clavicles, and reduced function of the diaphragm, which compromises respiration in offspring at birth and reduces neonatal survival. The three key events have been evaluated in a series of MoA studies aimed at examining the causality of sulfoxaflor's induction of these effects as observed in the regulatory guideline studies. The document represents the weight of evidence approach used to evaluate the data based upon the Bradford-Hill criteria followed by subsequent application in a Human Relevance Framework (HRF). The conclusion from this evaluation is that there is a high level of confidence that the observed sulfoxaflor-induced foetal abnormalities and neonatal offspring death in rats occur *via* a single MoA comprised of sustained activation of the rat foetal-type muscle nAChR resulting in muscle contracture. In addition, this MoA is not considered relevant to humans based upon available data demonstrating fundamental qualitative differences in sulfoxaflor agonism at the rat *versus* the human muscle nAChR where agonism occurs at the rat foetal-type, but not the human foetal or adult-type, muscle nAChR.

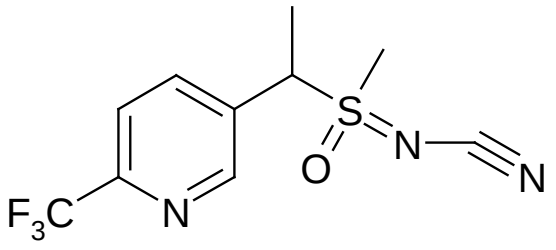
Summary

Sulfoxaflor, an insecticide that operates *via* the insect nicotinic acetylcholine receptor (nAChR), causes foetal abnormalities (primarily limb contractures) and death in neonatal rats, but not rabbits, following high dose dietary exposure during gestation in regulatory guideline studies. It is proposed that these effects have a single mode of action (MoA) mediated *via* the rat foetal-type muscle nAChR through the following key events: (1) binding to the receptor, (2) agonism (activation) at the receptor, causing (3) sustained muscle contracture in the near-term foetus and neonatal offspring. This sustained muscle contracture results in limb contractures, bent clavicles, and reduced function of the diaphragm, which compromises respiration in offspring at birth and

reduces neonatal survival. The three key events have been evaluated in a series of MoA studies aimed at examining the causality of sulfoxaflor's induction of these effects as observed in the regulatory guideline studies. This document represents the weight of evidence approach used to evaluate the data based upon the Bradford-Hill criteria followed by subsequent application in a Human Relevance Framework (HRF). The conclusion from this evaluation is that there is a high level of confidence that the observed sulfoxaflor-induced foetal abnormalities and neonatal offspring death in rats occur *via* a single MoA comprised of sustained activation of the rat foetal-type muscle nAChR resulting in muscle contracture. In addition, this MoA is not relevant to humans based upon available data demonstrating fundamental qualitative differences in sulfoxaflor agonism at the rat *versus* the human muscle nAChR where agonism occurs at the rat foetal-type, but not the human foetal or adult-type, muscle nAChR.

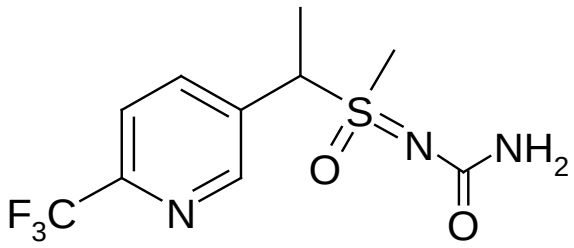
4.11.3.2. Data on Metabolites

Sulfoxaflor has been demonstrated to be an agonist on rat foetal-type (α1β1γδ) skeletal muscle nicotinic acetylcholine receptors (nAChRs). Two structurally related metabolites of sulfoxaflor (X11719474 and X11519540) were found to have no agonistic activity towards the rat foetal skeletal muscle nicotinic acetylcholine receptor and did not cause foetal abnormalities or neonatal death in rats. The inference is that these metabolites lack the functional group that enables binding or functional activation of the foetal receptor while being structurally very similar to the parent molecule sulfoxaflor.



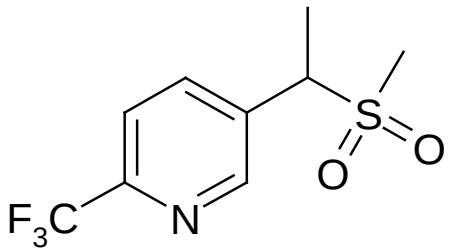
Parent: Sulfoxaflor

[methyl(oxo){1-[6-(trifluoromethyl)-3-pyridyl]ethyl}-λ4-sulfanylidene]cyanamide



Metabolite X11719474

N-(methyl(oxido){1-[6-(trifluoromethyl)pyridin-3-yl]ethyl}-λ4-sulfanylidene)urea



Metabolite X11519540

5-(1-methylsulfonyl)ethyl)-2-(trifluoromethyl)pyridine

4.11.4 Summary and discussion of reproductive toxicity

Relevant findings

There are four relevant findings with respect to reproductive toxicity;

1. Post-natal rat pup mortality and limb abnormalities;

2. A reduction in mean pup weights on PND1;

3. An increased post-implantation loss and decreased foetal weights in the main rat developmental toxicity study and;

4. A delay in preputial separation in the main rat 2 generation study.

1. Post natal rat pup mortality and limb abnormalities:

It has been demonstrated clearly during the conduct of a multigeneration study in the rat and developmental toxicity studies in the rat, that sulfoxaflor causes post-natal death and a specific grouping of limb abnormalities. No abnormalities were seen in developmental toxicity studies and a post-natal survival study in the rabbit. It has been proposed that these effects have a single mode of action (MoA) mediated *via* the rat foetal-type muscle nAChR through the following key events: (1) binding to the receptor, (2) agonism (activation) at the receptor, causing (3) sustained muscle contracture in the near-term foetus and neonatal offspring. This sustained muscle contracture results in limb contractures, bent clavicles, and reduced function of the diaphragm, which compromises respiration in offspring at birth and reduces neonatal survival. The hypothesis has been supported by a series of studies investigating the findings in the rat which have demonstrated that;

1.1 The effect of sulfoxaflor on pup survival was due to *in utero*, not lactational, exposure.

1.2 Sulfoxaflor was not developmentally toxic in the rabbit, despite the achievement of similar maternal and foetal systemic concentrations of sulfoxaflor in both rat and rabbit.

1.3 Sulfoxaflor has been shown to be a partial agonist of the rat foetal muscle nAChR. In contrast, sulfoxaflor has no detectable agonist activity on the human foetal muscle nAChR or on the adult forms of skeletal muscle nAChR (from either human or rat).

1.4 The critical period of developmental susceptibility to sulfoxaflor-induced foetal abnormalities and reduced neonatal survival is between GD 16-birth, and that the foetal structural abnormalities are rapidly reversible after birth in surviving pups.

1.5 The critical period of developmental susceptibility to sulfoxaflor-induced foetal abnormalities and reduced neonatal survival effects occurs shortly before birth.

NOTE:

The extensive data presented have gone a significant way towards identifying the MoA of the observed foetal mortalities and morphological alterations and have provided significant evidence that the MoA may not be relevant to man. However, there are some inconsistencies in the data and some information is lacking, therefore preventing the conclusion that the non-relevance to man has been proven with certainty.

- Sulfoxaflor was shown to have partial agonist activity in recombinant rat foetal muscle nAChR expressed in *Xenopus* ooctyes using a two-electrode voltage clamp procedure, while agonism was not detected in recombinant human foetal muscle nAChR, recombinant rat adult muscle nAChR, or recombinant human adult muscle nAChR. Preliminary results from a new study using recombinant (rat and human) receptors in HEK (Human Embryonic Kidney) cells confirm specific agonism of the rat foetal receptor only. However, rabbit

muscle nAChRs have not been examined due to technical difficulties in the molecular cloning of the rabbit muscle nAChR subunits, thus the lack of effect in the rabbit developmental toxicity study has not been investigated in functional receptor studies.

- The possibility of interaction with other cholinergic receptors (neuronal/nicotinic and muscarinic) has been considered. However, direct evaluations of sulfoxaflor agonism of neuronal receptors has not been conducted because clinical signs of such interactions have not been seen in adult rats or pups and because sulfoxaflor causes rigid contractures without evidence of receptor desensitisation (an effect more strongly associated with neuronal receptors). Clinical signs at birth of neuronal receptor mediated effects (post-natal respiratory distress) would be impossible to differentiate in the experimental data presented. However, it is noted that foetal lung histopathological analysis study showed that foetals lungs from the 1000 ppm sulfoxaflor treatment group (rat developmental toxicity study) were not different from control foetuses.

- The observation of reduced survival in the rat following gestional exposure from 400 ppm is consistent across a number of studies. Some inconsistencies exist in the data with regard to the foetal morphological findings. Such findings were not reported in the one-generation probe study at 1000 ppm (DAR B.6.6.1), although all pups were examined grossly for abnormalities. No sulfoxaflor mediated foetal abnormalities were noted at 1000 ppm in the probe developmental toxicity study in the rat (in which study foetuses were described as 'normal' (DAR B.6.6.10.1)). While it is stated that a detailed foetal examination was not carried out, any external abnormalities would/should have been noted. No pup morphological abnormalities were reported in the rat cross fostering study (DAR B.6.6.12.1) even though all (caesarean-sectioned) pups were examined grossly. Convoluted ureters and bent clavicles were not seen in the critical window studies at the same doses that caused these effects in the developmental toxicity study (DAR B.6.6.12.4-5). This may be related to reversibility of these effects as discussed in the study summary.

- It ia noted that the structure of sulfoxaflor leads to specific binding to the rat foetal nAChR with associated post-natal mortality and structural alterations, an effect not previously demonstrated for other structurally related neonicotinoid pesticidal substances. This difference is considered to be related to its novel chemical structure, and the unique way in which sulfoxaflor binds with the insect nAChR (different to previous neonicotinoids). Additionally, sulfoxaflor is metabolised very little unlike other related chemicals.

The question remains whether sufficient proof has been provided of non-relevance to humans of this substance-related post-natal death and the structural abnormalities demonstrated to occur in the rat and not seen in the rabbit. Detailed technical examination of the evidence is recommended before the definitive classification can be made by the appropriate authority. The conclusion is that the case for non classification can be supported on the basis of the data presented for the pharmacologically mediated effects.

2: Reduction in mean pup weights on PND1

In addition to the findings related to the pharmacological action of sulfoxaflor, there was a reduction in mean pup weights in a number of studies: at PND1 in the reproduction probe study (DAR B.6.6.1) at 1000 ppm; in the developmental neurotoxicity study (DNT) (DAR B.6.7.2) where mean pup body weights in the 400 ppm group were 11.8% and 6.5% lower than the control group at PND 1 and on PND 4, respectively; in the cross-fostering study (DAR B.6.6.12.1) where PND1 pup weights were significantly lower at 1000 ppm, but PND0 mean pup weights were not

different from control. In the DNT study, this reduction in body weight was associated with a statistically significant delay in surface righting response for pups in the 400 ppm group.

3: Increased post-implantation loss and decreased foetal weights.

There was evidence of foetotoxicity in the main rat developmental study where increased postimplantation loss, and decreased foetal weights were noted at the high dose. The adverse foetal findings at this dose level (1000 ppm (70.2 mg/kg bw/day) were associated with significant maternal toxicity i.e., decreased mean body weight and mean body weight gains.

Table 4.11.4. Summary-1 (DAR B.6.6.10.2-3): Caesarean Section Observations)

Dose (ppm)	0	25	150	1000
Number Bred	26	26	26	26
% Pregnant [a]	24/26 (92.3%)	23/26 (88.5%)	25/26 (96.2%)	25/26 (96.2%)
Number of Deaths	0	0	0	0
Number Moribund	0	0	0	0
Number Aborted	0	0	0	0
Number Removed Early	0	0	0	0
Pregnancies Detected by Stain [b]	0/2	0/3	0/1	0/1
Number of Litters Totally Resorbed	0	0	0	0
Number of Litters with Viable Foetuses	24	23	25	25
Number of Corpora Lutea/Dam [c]	14.1 ± 1.9	14.1 ± 1.7	14.3 ± 1.4	13.5 ± 1.6
Number of Implantations/Dam [c]	13.5 ± 1.8	13.3 ± 1.6	13.9 ± 1.5	13.0 ± 1.5
Mean % Preimplantation Loss [d]	3.6 ± 5.8	5.6 ± 7.0	2.8 ± 4.4	3.4 ± 6.3
Number of Resorptions/Litter [c,f]	0.2 ± 0.4	0.7 ± 1.0	0.6 ± 1.0	0.7 ± 0.9
Resorptions/Litters with Resorptions [f]	1.0 (5/5)	1.7 (15/9)	1.8 (16/9)	1.5 (18/12)
Mean % Postimplantation Loss [e]	1.4 ± 2.8	4.9 ± 7.8	5.1 ± 8.1	**5.2 ± 6.4**
Viable Foetuses/Litter [c]	13.3 ± 1.7	12.7 ± 1.9	13.3 ± 2.1	**12.3 ± 1.3[$]**
Foetal Weight – Males (g) [c]	5.94 ± 0.31	6.02 ± 0.31	6.02 ± 0.25	**5.29 ± 0.32***
Foetal Weight – Females (g) [c]	5.67 ± 0.26	5.71 ± 0.37	5.63 ± 0.28	**4.99 ± 0.27***
Foetal Weight – Sexes Combined (g) [c]	5.79 ± 0.26	5.87 ± 0.34	5.83 ± 0.25	**5.12 ± 0.30***
Gravid Uterine Weight (g) [c]	106.38 ± 12.18	102.80 ± 13.48	106.62 ± 15.17	**92.34 ± 10.00***
Sex Ratio (M:F)	48:52	52:48	49:51	44:56

[a] No. of Females With Visible Implantations/Total No. Bred.

[b] No. of Females Detected as Being Pregnant After Sodium Sulfide Stain/Total No. Stained.

[c] Mean ± S.D.

[d] Mean Percent/Litter (Calculated As [(No. Corpora Lutea - No. Implantations)/No. Corpora Lutea] X 100

[e] Mean Percent/Litter (Calculated As [(No. Implantations – Live Born Pups / No Implantations] X 100

[f] Not Statistically Analyzed.

$ Statistically Different from Control Mean by Wilcoxon's Test, Alpha=0.05.

* Statistically Different from Control Mean by Dunnett's Test, Alpha=0.05.

Table 4.11.4. Summary-2 (DAR Table B.6.6.10.2-4) Caesarean Section Observations Historical Control gavage studies

	1 6/2004	2 7/2004	3 6/2005	4 8/2005	5 10/2005	6* 8/2005	7* 2/2009
Mean % Post implantation Loss	3.9 ± 7.5	3.6 ± 4.9	1.2 ± 3.7	7.0 ± 8.3	7.1 ± 9.9	8.2 ± 12.6	5.2 ± 6.9
Viable Foetuses/Litter	12.9 ± 2.4	13.0 ± 1.6	13.0 ± 2.2	12.2 ± 2.1	12.3 ± 2.1	12.6 ± 1.9	11.0 ± 3.7

*Data collected from probe studies.

Data extracted from pg 26 of the study report.

Table 4.11.4. Summary-3 (B.6.6.10.2-2): Maternal Body Weights Summary (grams))

		Day of Gestation							
DOSE PPM		**0**	**6**	**9**	**12**	**15**	**18**	**21**	**21[a]**
0	Mean	236.0	272.5	288.0	311.2	331.7	372.9	425.4	319.0
	S.D.	7.3	11.7	15.2	17.1	21.0	20.8	27.2	23.2
	N=	24	24	24	24	24	24	24	24
25	Mean	232.9	270.6	286.3	309.0	329.1	371.2	420.7	317.9
	S.D.	6.4	9.8	10.6	12.9	14.3	15.9	20.4	20.3
	N=	23	23	23	23	23	23	23	23
150	Mean	234.0	272.6	289.9	312.2	332.3	374.1	426.6	319.9
	S.D.	6.0	8.3	10.3	11.8	13.6	16.5	20.3	18.7
	N=	25	25	25	25	25	25	25	25
1000	Mean	234.9	268.6	269.4* (↓7%)	288.4* (↓7%)	306.7* (↓7%)	344.8* (↓7%)	387.2* (↓9%)	294.9* (↓7%)
	S.D.	7.3	12.0	11.0	13.1	14.5	17.2	18.7	16.4
	N=	25	25	25	25	25	25	25	25

* Statistically different from control mean by Dunnett's test, alpha=0.05.
a = terminal body weight - gravid uterine weight
(%) indicates percent change compared to concurrent control group

Table 4.11.4. Summary-4 (DAR Table B.6.6.10.2-1.): Maternal Body Weight Gain Summary (grams))

		Days of Gestation							
Dose PPM		**0-6**	**6-9**	**9-12**	**12-15**	**15-18**	**18-21**	**6-21**	**0-21**
0	Mean	36.6	15.5	23.2	20.5	41.2	52.5	152.9	189.4
	S.D.	6.9	6.0	6.1	5.4	5.8	9.3	18.5	22.4
	N=	24	24	24	24	24	24	24	24
25	Mean	37.8	15.7	22.7	20.2	42.1	49.5	150.1	187.8
	S.D.	6.2	3.6	6.8	4.4	5.5	12.6	18.2	19.2
	N=	23	23	23	23	23	23	23	23
150	Mean	38.5	17.3	22.3	20.1	41.8	52.5	154.0	192.5
	S.D.	6.9	5.1	5.3	6.3	7.2	6.1	17.1	19.5
	N=	25	25	25	25	25	25	25	25
1000	Mean	33.8	0.8[$]	19.0	18.3	38.1	42.4[$]	118.6* (22%)	152.4* (20%)
	S.D.	10.0	8.4	7.6	5.5	8.5	5.7	15.2	18.3
	N=	25	25	25	25	25	25	25	25

* Statistically different from control mean by Dunnett's test, alpha=0.05.
$ Statistically different from control mean by Wilcoxon's test, alpha = 0.05
(%) indicates percent change compared to concurrent control group

4: Delayed preputial separation.

In the main 2-generation study, there was also an apparent treatment-related delay in preputial separation (PPS) for 400 ppm F_1 males. This external marker of male puberty onset is androgen dependent, but the underlying reason for induction of this finding by sulfoxaflor is not known; however, there were no other indications of androgenic or anti-androgenic effects. Taken together,

the weight of evidence across androgen-sensitive endpoints led to the conclusion that the data do not support any other sulfoxaflor-mediated anti-androgenic effects. Specific mode of action studies (DAR B.6.5.4.3) investigated oestrogen receptor and androgen receptor agonism and antagonism in addition to aromatase inhibition. The results were negative and do not support biological effects mediated *via* the sex hormone receptors.

4.11.5 Comparison of the relevant findings with CLP and DSD classification criteria

DSD:

Cat 1:

- Human evidence (epidemiological) is required for classification in this category, therefore sulfoxaflor does not classify as Cat1.

Cat 2/Cat 3:

- The adverse effects on pup survival and structural alteration in the rat would normally fulfil the category for classification in Cat 2 or 3 for development. However, mechanistic evidence has been presented that the observed effects are related to the pharmacological action of sulfoxaflor and are species specific. It has been proposed that this effect is not relevant to man due to specific differences in the subunit structure of the muscle nAChR between man, rat and rabbit and differences therefore in the binding and functional activation of the receptor complex by sulfoxaflor.

  The criteria state '*..even when clear effects have been demonstrated in animal studies, the relevance to humans may be doubtful because of the doses administered, for example, where effects have been demonstrated only at high doses, or when marked toxicokinetic differences exist, or the route of administration is inappropriate.* ***For these or other reasons*** *it may be that classification in Cat 3****, or even no classification, will be warranted'***.

  The extensive mechanistic evidence has provided significant support for the non-relevance to humans of the pharmacological effects, but with some inconsistancies and data gaps as described above. In balance, the evidence is considered to support non-classification for this effect, but it is recognised that in depth discussion will be necessary on this point.

- The apparent increase in post-implantation loss and reduction in foetal body weight in the rat (at 1000 ppm/70 mg/kg bw) in the main developmental toxicity study in the rat were seen in conjunction with significant maternal body weight effects. The apparent increase in post-implantation loss was not dose-related and was within the recent historical control range and unlikely to be treatment-related. In addition, the post-implantation loss in the concurrent control was at the lower end of the historical control data range. This finding does not support classification. The clear reduction in foetal weights seen at 1000 ppm in this study is most likely treatment-related and evidence of a foetotoxic effect. However, maternal toxicity was apparent at this high dose and the effect is considered borderline and may not be supportive of classification. It is noted that no such effect was seen in the rabbit studies.

  A significant reduction in mean pup body weights was seen in the 400 ppm group (11.8% and 6.5% lower than the control group (PND 1) and on PND 4, respectively) of the developmental neurotoxicity study and in the cross-fostering study (B.6.6.12.1) in litters exposed *in utero* to sulfoxaflor (1000 ppm). The reduced pup body weights were associated with a statistically significant delay in surface righting response for pups in the 400 ppm group in the DNT study. These findings occurred at doses that impaired survival and are

likely to be related to toxicity imposed by the pharmacological action of the molecule (and therefore rat specific), i.e., breathing difficulties and an inability to move and nurse normally resulting in loss of weight by the end of PND1. It is noted that pup weights were not different from controls immediately following birth and were reduced by PND1 in the cross-fostering study (see Table B.6.6.12.1-5b). This effect is likely to reflect the pharmacological action of the substance and as such considered non-relevant to man.

There was an apparent treatment-related (statistically significant) delay (2.4 days) in puberty onset and preputial separation in F1 males of the 2-generation. The attainment of sexual developmental landmarks (balanopreputial separation and vaginal patency) was unaffected at the same dose levels in the developmental neurotoxicity study (B.6.7.5). In addition, there is no other evidence of an anti-androgenic effect of sulfoxaflor and this finding is not considered supportive of classification.

CLP:

Classification under the CLP regulation criteria is not proposed on the basis of the same findings. Unlike the DSD criteria, the CLP emphasises the issue of mechanistic data stating '*...However, where mechanistic data raises doubt about the relevance of the effect for humans, classification in Cat 2 may be more appropriate.*' Good quality mechanistic data has been generated which goes beyond the criteria which state '*raises doubt about the relevance of the effect to man*'..., and supports the human non-relevance of the effect on post-natal survival and skeletal alterations; therefore classification is not supported on the basis of this endpoint.

The reduced pup weights on PND1 in the rat developmental neurotoxicity study (also delayed righting reflex in the neurotoxicity study) at 400 ppm, at 1000 ppm in the reproduction probe study (B.6.6.1) and in the cross-fostering study were not associated with significant maternal toxicity. However, reduced PND1 pup body weights occurred at doses causing reduced survival and are likely to be related to toxicity imposed by the pharmacologic action of the molecule (and rat specific), i.e., breathing difficulties and an inability to move and nurse normally, resulting in loss of weight by the end of PND1. It is noted that pup weights were not different from controls immediately following birth and were reduced by PND1 in the cross-fostering study (see Table B.6.6.12.1-5b).

The finding of reduced pup weight in the rat developmental study was considered treatment-related but also associated with significant maternal toxicity. No such effect was seen in the rabbit studies presented in the substance dossier. According to the criteria, an adverse effect on development not sufficiently convincing to place the substance in Cat 1, should be considered for Cat 2. As significant maternal toxicity was seen at 1000 ppm also causing a reduction in mean foetal body weight, it is considered that the toxic effect may be 'a secondary non-specific consequence of other toxic effects'. This conclusion is supported by the lack of effect in the second species, the rabbit.

4.11.6 Conclusions on classification and labelling

Relevant mechanistic data was submitted that provided significant support for the non relevance to humans of the proposed rat-specific adverse effect. Therefore, classification in Cat 3 (DSD) or Cat 2 (CLP) is not supported. Foetotoxicity expressed as reduced foetal weight in the rat developmental study was associated with maternal toxicity and not seen in the rabbit. The significantly reduced PND1 weight (and delayed righting reflex in DNT study) occurred in the rat in a number of studies and is likely to be related to the pharmacological action of the molecule (breathing difficulties and an inability to move and nurse normally resulting in loss of weight by the end of PND1) and therefore not relevant to humans. There were no adverse findings in the rabbit.

In conclusion, the data submitted when considered in its entirety, provides a strong argument for non-classification, however, it is recognised that an in depth discussion will be necessary on this endpoint (developmental toxicity).

4.12 Other effects

4.12.1 Human information

No data.

4.12.2 Non-human information

Not relevant.

4.12.2.1 Specific investigations: other studies

No additional data.

4.12.2.2 Human information

None available.

4.12.3 Summary and discussion of other effects

4.12.4 Comparison with CLP and DSD classification criteria

Conclusions on classification and labelling4.12.3 – 4.12.5 are not relevant in this evaluation

5 ENVIRONMENTAL HAZARD ASSESSMENT

The data presented in this section is reproduced directly from the **Draft Assessment Report (DAR) for Sulfoxaflor** either in summary form or as robust study summeries, as appropriate. The Draft Assessment Report (DAR) for Sulfoxaflor is prepared in accordance with Reg. (EC) No. 1107/2009 concerning the placing of Plant Protection Products on the market.

In addition, to the relevant CLH report numbering DAR references are also given for each endpoint. In the case of endpoints that are relevant for hazard identification according to CLP and DSD criteria the text is reproduced directly from the Draft Assessment Report (DAR) for Sulfoxaflor. In this case, the study will be headed Study X (Sulfoxaflor DAR, XX sections B.6.X.x.X). The details in brackets will indicate the original location of the data in the DAR. It is also necessary to point out that the figures and tables will be adapted to indicate the CLH report and DAR dual numbering. The in-text citations will remain as they were for the DAR and will not be adapted to match with the CLH report.

5.1 Degradation

Table 5.1-1: Summary of relevant information on degradation

Method	Results	Remarks	Reference
OECD 111 – Hydrolysis as a function of pH	Sulfoxaflor is hydrolytically stable in water in the whole environmentally relevant range of pH (5-9)	none	Laughlin L. A, 2009;
OECD 316 – Phototransformation of Chemicals in Water – Direct Photolysis; US EPA OPPTS 835.2240 Photodegradation in Water; Study performed using sterilised buffer solutions to examine direct photolysis of sulfoxaflor in aqueous solutions.	Sulfoxaflor does not undergo direct photolysis in aquatic environment	The results of the study indicated some photodegradation, but the evaluator decided that most probably this was an artifact due to the inadequate selection of the buffer	Ma M.; 2011
OECD 316 – Phototransformation of Chemicals in Water – Direct Photolysis; US EPA OPPTS 835.2240 Photodegradation in Water; Study performed using natural, not sterilised water, to examine direct and indirect aqueous photolysis of sulfoxaflor.	Sulfoxaflor does not undergo indirect photolysis in aquatic environment	none	Yoder R. N.; 2010
OECD 310 – Ready Biodegradability – CO_2 in Sealed Vessels (Headspace Test); ISO 14593, Water Quality – Evaluation of ultimate aerobic biodegradability of organic compounds in aqueous medium	Sulfoxaflor is not ready biodegradable – after 28 days less than 3% was transformed into CO_2	none	Fiel N.; 2010

OECD 308 – Aerobic and Anaerobic Transformation in Aquatic Sediment System; US EPA OPPTS 835-4300 Aerobic Aquatic Metabolism	Sulfoxaflor was confirmed to be not ready biodegradable – after 103 days mineralisation level was 0.5- 1.5% of the applied; Sulfoxaflor was demonstrated not to be rapidly biotically degradable in the aquatic environment (water/sediment system) – DT_{50} = 57.08 days (37.67 -88.86 days); DT_{90} = 189.63 days (121.83 – 295.20 days). The degradation mechanism was predominantly biotic, the degradation product was X11719474 (stable)	none	Laughlin L. A., Adelfinskaya Y., Balcer J. L.; 2010

5.1.1 Stability

In order to determine the stability (fate and behaviour) of Sulfoxaflor in the aquatic environment the following processes were examined: abiotic hydrolysis, direct and indirect photolysis in the aquatic environment. The studies were performed according to the relevant OECD Gudelines – 111 for aqueous hydrolysis and 316 for aqueous photolysis. No significant deviations were stated, therefore the studies were considered acceptable, hence reliable for the determination of the stability of Sulfoxaflor in the aquatic environment.
They are briefly summarized below.

The examination of the aqueous abiotic hydrolysis of Sulfoxaflor (**Laughlin A, 2009; study report No. 070102**), performed in an environmentally relevant range of pH (in three sterile buffer solutions at pH 5, pH 7 and pH 9) demonstrated that the concentration of Sulfoxaflor in the buffer solution remained practically unchanged. Therefore it was concluded that Sulfoxaflor is hydrolytically stable in the whole range of the environmentally relevant pH and the proposed DT_{50} values for the abiotic hydrolysis in water are 1000 days for the whole pH range of 5-9.

Direct aqueous photolysis was examined in sterile buffer solution (TRIS buffer) at pH 7 and T = 25^0C (**Ma M., 2011; study report No. 09007**). The process was examined for Sulfoxaflor and its major metabolite – X11719474. It was stated that both compounds underwent slow photodegradation. The determined kinetic endpoints, not corrected for the lamp intensity were following:

- for Sulfoxaflor DT_{50} = 489 days, DT_{90} > 1000 days;
- for X11719474 DT_{50} = 136 days, DT_{90} = 451 days.

When recalculated as a function of latitude and season, these values for a summer day at the latitude of 40N were following:

- for Sulfoxaflor DT_{50} = 7500 days, DT_{90} = 24915 days;
- for X11719474 DT_{50} = 261 days, DT_{90} = 868 days.

Two photodegradation products, both minor were identified in the study – X11721061 for both Sulfoxaflor and X11719474 and X11718922 for X11719474 only.

These results should be considered with extreme caution, as neither Sulfoxaflor nor X11719474

absorb the UV-Vis radiation in the environmentally relevant range of λ = 290-800 nm. Therefore the following conclusion with regard to direct aqueous photolysis is proposed:

Neither Sulfoxaflor nor X11719474 are expected to undergo direct aqueous photolysis, due to the lack of absorption of the UV-Vis radiation in the environmentally relevant wavelength range – λ = 290 – 800 nm. Therefore direct photolysis cannot be regarded as a relevant degradation mechanism for either Sulfoxaflor or X11719474 in the aquatic environment.

The examination of the aqueous photolysis in natural water (direct and indirect) was examined for both Sulfoxaflor and X11719474 (**Yoder R. N., 2010; study report No. 090088**). The determined kinetic endpoints, not corrected for the lamp intensity were following:

- for Sulfoxaflor DT_{50} = 224 days, DT_{90} = 743 days;
- for X11719474 DT_{50} = 444 days, DT_{90} > 1000 days.

When recalculated as a function of latitude and season, these values for a summer day at the latitude of 40^0N were following:

- for Sulfoxaflor DT_{50} = 637 days, DT_{90} > 1000 days;
- for X11719474 DT_{50} > 1000 days, DT_{90} > 1000 days.

No identifiable photodegradates of either Sulfoxaflor or X11719474, major or minor, are expected to occur as a result of this process. It can be stated that aqueous photolysis, either direct or indirect should not be regarded as a relevant degradation mechanism for either Sulfoxaflor or X11719474 in surface water bodies.

The detailed results of the examination of stability (abiotic degradation) of Sulfoxaflor in the aquatic environment are presented in the table below.

Table 5.1.1-1: The detailed results of the examination of stability of Sulfoxaflor in the aquatic environment.

Process	Experimental conditions	Obtained results	
		Degradation kinetics	***Identified metabolites***
Abiotic hydrolysis	pH = 5 (sterile acetate buffer); T = 25^{0}C; incubation in the absence of light (darkness); test substance: ^{14}C-Sulfoxaflor	DT_{50} > 1000 days – compound hydrolytically stable at this pH	None detected - compound hydrolytically stable at this pH
	pH = 7 (sterile TRIS buffer); T = 25^{0}C; incubation in the absence of light (darkness); test substance: ^{14}C-Sulfoxaflor	DT_{50} > 1000 days – compound hydrolytically stable at this pH	None detected - compound hydrolytically stable at this pH
	pH = 9 (sterile borate buffer); T = 25^{0}C; incubation in the absence of light (darkness); test substance: ^{14}C-Sulfoxaflor	DT_{50} > 1000 days – compound hydrolytically stable at this pH	None detected - compound hydrolytically stable at this pH
Aqueous photolysis in sterile buffered solution (direct aqueous photolysis)	Sterile TRIS buffer (pH 7); Xenon lamp working at the wavelength range λ = 290-800 nm as a light source; intensity of light 300 W/m^2; incubation temperature T = 25^{0}C; dark control samples and actinometers set alongside irradiated samples; test compounds: ^{14}C-Sulfoxaflor and ^{14}C-X11719474; study duration: 14 days	Sulfoxaflor: DT_{50} = 7500 days, DT_{90} = 24915 days (average summer day at 40N); X11719474: DT_{50} = 261 days, DT_{90} = 868 days (average summer day at 40N); The compounds are not prone to direct photolysis in the aquatic environment. Quantum yield Φ could not be determined – none of the test substances absorbed UV-Vis radiation in the environmentally relevant wavelengths range λ = 290-800 nm.	None (minor photodegradation products X11721061 and X1171892 are probably the products of the indirect photolysis related to the use of TRIS buffer).
Aqueous photolysis in natural water (direct and indirect aqueous photolysis)	Natural lake water (pH = 8.2); Xenon lamp working at the wavelength range λ = 290-800 nm as a light source; intensity of light 300 W/m^2; incubation temperature T = 25^{0}C; dark control samples and actinometers set alongside irradiated samples; test compounds: ^{14}C-Sulfoxaflor and ^{14}C-X11719474; study duration: 14 days	Sulfoxaflor: DT_{50} = 637 days, DT_{90} > 1000 days (average summer day at 40N); X11719474: DT_{50} > 1000 days, DT_{90} > 1000 days (average summer day at 40N); The compounds are not prone to photolysis, direct or indirect in the aquatic environment.	None identified

5.1.2 Biodegradation

Biodegradation of Sulfoxaflor was examined in two separate studies:

a) Study on ready biodegradability (**Fiel N., 2010; study report No. 54631082**);

b) Water sediment study (**Laughlin L. A, Adelfinskaya Y., Balcer J. L, 2010; study report No. 080138**).

5.1.2.1 Biodegradation estimation

Sulfoxaflor was demonstrated to be not ready biodegradable – in the experiment on ready biodegradability as less than 3% of the applied compound underwent mineralization after 28 days, while in water/sediment study the mineralization level after 103 days was 0.6 -1.5% of the applied dose. It was also demonstrated that this compound cannot be considered as rapidly biologically degradable – the geomean DT_{50} in the aquatic systems was 57.08 days, what indicates that within 28 days much less than70% of its amount would undergo the biotic degradation.

5.1.2.2 Screening tests

The ready biodegradability of Sulfoxaflor was examined in the headspace test (**Fiel N., 2010; study report No. 54631082**). The study was performed according to the OECD Guideline 310. It was evaluated and no significant deviations were noted. Therefore the study was accepted and considered reliable for the assessment of ready biodegradability of sulfoxaflor. It was stated that only up to 2.5% of applied Sulfoxaflor was mineralised within 28 days, while the mineralization of the reference compound – sodium benzoate, was complete. **Therefore Sulfoxaflor shall be regarded as not ready biodegradable.**

The summary of this study, as presented in the Draft Assessment Report for Sulfoxaflor, is given below.

Study 1:

Report: Fiel N., (2010): "Ready Biodegradability of XDE-208 technical in a CO_2 Headspace Test.". Institut für Biologische Analytik und Consulting IBACON GmbH, Arheilger Weg 17, 64380 Rossdorf, Germany, *for* Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, Indiana 46268-1054, USA; unpublished study report No. 54631082; 18 May 2010.

Guidelines: study was carried out to comply with the following:

- OECD Guideline for the Testing of Chemicals 310 – Ready Biodegradability – CO_2 in Sealed Vessels (Headspace Test); Guideline adopted on 23rd March 2006;
- ISO 14593, Water Quality – Evaluation of ultimate aerobic biodegradability of organic compounds in aqueous medium – Method by analysis of inorganic carbon in sealed vessels (CO_2 headspace test), March 15, 1999;

GLP: Yes.

Comments: The study was evaluated using the following guidelines of those listed above:

- OECD Guideline for the Testing of Chemicals 310 – Ready Biodegradability – CO_2 in Sealed Vessels (Headspace Test); Guideline adopted on 23rd March 2006.

The study was evaluated, it is acceptable.

Summary:

The aim of the study was to determine the aerobic ready biodegradability of the technical sulfoxaflor (~95% chemical purity) in a CO_2 Headspace Test.

The experiment was performed in a way to comply with the following Guidelines:

- OECD 310 Guideline: Ready Biodegradability: CO_2 in Sealed Vessels (Headspace Test),
- ISO 14593 Guideline: Water Quality – Evaluation of ultimate aerobic biodegradability of organic compounds in aqueous medium – Method by analysis of inorganic carbon in sealed vessels (CO_2 headspace test).

It was stated that no deviations from the Guidelines were observed, hence none reported.

The test substance was the non-radiolabelled, technical sulfoxaflor, having the chemical purity of 95.6% (determined using HPLC). It was supplied to the test-performing facility as a white solid substance.

The reference compound was sodium benzoate having the chemical purity of 100% (according to the data provided by the manufacturer of this substance).

Both compounds were stored, until being used in the original containers in the dark, sulfoxaflor at temperatures T = 5 – 25^{0}C and sodium benzoate at room temperature (T = 20 ± 5^{0}C).

The experiment on ready biodegradability was performed using the activated sludge from a domestic waste water treatment plant, obtained from municipal sewage treatment plant in

Darmstadt, Germany. The sludge suspension, after the determination of its dry matter content, was aerated using CO_2-free air up to 1 day before being used.

The test vessels were 125 mL (nominal volume; total volume – 128 mL) glass flasks with screw-caps and Teflon-coated septa. Each flask was individually marked.

The liquid test medium was prepared in a following way:

First the following stock solutions of analytical grade salts were prepared:

- **Solution 1:** 8.5 g KH_2PO_4, 21.75 g K_2HPO_4, 33.4 g $Na_2HPO_4 \cdot 2\ H_2O$ and 0.5 g NH_4Cl were dissolved in deionised water in volumetric flask to 1000 mL volume;
- **Solution 2:** 22.5 g $MgSO_4 \cdot 7\ H_2O$ was dissolved in deionised water in volumetric flask to 1000 mL volume;
- **Solution 3:** 36.4 g $CaCl_2 \cdot 2\ H_2O$ was dissolved in deionised water in volumetric flask to 1000 mL volume;
- **Solution 4:** 0.25 g $FeCl_3 \cdot 6\ H_2O$ was dissolved in deionised water in volumetric flask to 1000 mL volume; to avoid the precipitation of $FeOH_3$ in the stock solution, it was acidified with concentrated HCl_{aq} (one drop per 1 L of solution).

The test medium solution was prepared by combining 10 mL of the **Solution 1** with **Solutions 2 - 4** (1 mL of each) in 1 L. volumetric flask and filling in with purified deionised water up to 1000 mL. So prepared test medium solution was used to prepare **test water** by mixing it with the appropriate amount of the activated sludge to get a final concentration of 4 mg dry material/L.

Finally the following test solutions were prepared:

- **Test Item solution (F_T)** prepared by direct dissolving the appropriate weighed amount of the test compound – sulfoxaflor (sulfoxaflor is readily dissolved in water), in 2500 mL of test water, to get the concentration of the sulfoxaflor 46 mg/L (corresponding to the carbon concentration in solution 20 mg/L ± 15%); so prepared samples were then dispensed into single test vessels which were next sealed with gas-tight septum caps;
- **Procedure Control solution (F_C)** prepared by first dissolving the reference compound – sodium benzoate, in purified deionised water to get the stock solution, the aliquots of which were then mixed with the test water to get the concentration of sodium benzoate 34 mg/L (corresponding to the carbon concentration in solution 20 mg/L ± 15%); so prepared samples were then dispensed into test vessels which were next sealed with gas-tight septum caps;
- **Inoculum control solution (F_B):** the aliquots of test water without any additives were dispensed into test vessels which were next sealed with gas-tight septum caps;
- **Toxicity control solution (F_I)** prepared by first dissolving the reference compound – sodium benzoate in the appropriate amount of test water; this solution was thoroughly mixed and its appropriate amounts were dispensed into the test vessels; to these solutions the appropriate amounts of the test compound – sulfoxaflor were added in a way identical to that described for the preparation of the solution F_T; so prepared final solution contained 23 mg sulfoxaflor/L and 17 mg sodium benzoate/L (corresponding to the total carbon concentration in solution of 20 mg/L ± 15%); so prepared test vessels were then sealed with gas-tight septum caps;

The headspace:liquid ratio in so prepared test vessels was 1:2. Then the test vessels were placed in the darkness in a constant temperature T = 20 ± 1^0C and incubated up to 28 days. The sampling points were set to DAT 0, 2, 5, 7, 9, 14, 21 and 28 for F_T and F_B solutions and DAT 0, 5, 14 and 28

for $\mathbf{F_C}$ and $\mathbf{F_I}$ solutions. For these sampling points three replicates of each tested solution were prepared, with exception of DAT 28, for which five replicates of each tested solution were prepared.

To the each test vessel removed from the incubation chamber the appropriate amount of 7M NaOH was injected (e.g. 0.8 mL to 85 mL test medium) to convert the produced CO_2 into carbonates. Then the alkalised solutions, after approximately 1-hour shaking, were analysed using TIC method. This was done using the TOC (Total Organic Carbon) Analyser (TOC-V CHP, Shimadzu) equipped with autosampler (ASI-V, Shimadzu) and IR Gas Analyzer as a detector. The carrier gas was oxygen. The samples were first acidified to pH <3 with a small amount of 40% H_3PO_4 in order to re-convert carbonates into CO_2, then the CO_2-free air was passed through the samples in order to volatilise all CO_2 present in them (including the dissolved CO_2). This air stream was directed into the analyser.

If it was not possible to analyse samples immediately after alkalinisation, they were stored deep frozen ($\leq -10^0$C) up to 15 weeks before being analysed.

The analysis of the IC content in samples was performed using the calibration curve. This curve was built using a set of calibration solutions, made of a stock aqueous solution of either $NaHCO_3$ (1.750 g, dried overnight in silica gel dessicator, dissolved in 500 mL of pure water) or Na_2CO_3 (2.205 g, dried for 1 hour at 280^0C and cooled in a silica gel dessicator, dissolved in 500 mL of pure water). Carbon concentration of the stock solution was 1000 mg C/L. The calibration standards were in range of 1 mg C/L – 25 mg C/L.

The LOD level was determined mathematically from the linear calibration curves, while the LOQ level for the test item was determined as the lowest fortification level at which the acceptable recovery – 70 - 110% of nominal, was obtained.

The level of biodegradation (in %) was calculated using the following equation:

$$\%D = (IC_{produced}/ThCO_2) * 100$$

where:
%D is percentage degradation of either test compound or reference compound (sodium benzoate);
$\mathbf{IC_{produced}}$ is the quantity of inorganic carbon (in form of CO_2, expressed in mg/L) measured in samples as produced from either test compound or the reference compound;
$\mathbf{ThCO_2}$ is the theoretical CO_2 production, in mg CO_2/L, i. e. the quantity of CO_2 that might be produced were the compound totally mineralised; this is calculated from the known or measured carbon content of the given compound.

Following validity criteria were set for the experiment:

- the IC content in the test item suspension in the mineral medium ($\mathbf{F_T}$) at the beginning of the study should be < 15% TC (total carbon content);
- the mean amount of the TIC (total inorganic carbon) in the blank control samples ($\mathbf{F_B}$) should be < 3 mg C/L;
- the **%D** of the reference item (sodium benzoate) must reach the level for ready biodegradability (>60%) by DAT 14.

Results and their discussion:

The method's LOD was not given, while the LOQ for the test compound was determined to be 1 mg/L (at this fortification level mean recovery level was 87%). The results of the experiment are presented below in the table 5.11.2.4-1 (DAR Table B.8.4.3.1-1).

Table 5.1.2.4-1 (DAR Table B.8.4.3.1-1): Results of the experiment presented in form of the amount of the inorganic carbon (IC) in mg/L produced during the test period.

Results obtained for F_T solution (*Test Item solution*, containing sulfoxaflor):												
DAT[1)]	***TIC [mg/L][2)]***						***Corrected TIC [mg/L][3)]***					
	Replicate					Average	Replicate					Average
	1	2	3	4	5		1	2	3	4	5	
0	0.89	0.87	n. r[4)]	n. a.[5)]	n. a.[5)]	0.58	0.07	-0.54	n. a.[5)]	n. a.[5)]	n. a.[5)]	-0.24
2	-0.42	-0.72	0.10	n. a.[5)]	n. a.[5)]	-0.35	-0.24	-0.54	0.28	n. a.[5)]	n. a.[5)]	-0.17
5	0.88	0.81	1.05	n. a.[5)]	n. a.[5)]	0.91	-0.18	-0.24	-0.01	n. a.[5)]	n. a.[5)]	-0.14
7	1.09	1.28	n. r[4)]	n. a.[5)]	n. a.[5)]	1.18	0.25	0.44	n. a.[5)]	n. a.[5)]	n. a.[5)]	0.34
9	1.01	1.05	0.95	n. a.[5)]	n. a.[5)]	1.00	0.24	0.28	0.18	n. a.[5)]	n. a.[5)]	0.23
14	2.15	2.63	2.12	n. a.[5)]	n. a.[5)]	2.30	0.02	0.50	-0.01	n. a.[5)]	n. a.[5)]	0.17
21	1.91	1.85	1.86	n. a.[5)]	n. a.[5)]	1.87	0.29	0.23	0.24	n. a.[5)]	n. a.[5)]	0.25
28	n. r[4)]	2.51	1.75	n. r[4)]	2.27	2.81	n. a.[5)]	0.02	-0.74	n. a.[5)]	-0.22	-0.31
Results obtained for F_C solution (*Procedure Control solution*, containing reference compound- sodium benzoate):												
DAT	***TIC [mg/L][2)]***						***Corrected TIC [mg/L][3)]***					
	Replicate					Average	Replicate					Average
	1	2	3	4	5		1	2	3	4	5	
0	0.73	0.18	0.92	n. a.[5)]	n. a.[5)]	0.61	-0.08	-0.63	0.10	n. a.[5)]	n. a.[5)]	-0.20
5	29.44	25.83	33.0	n. a.[5)]	n. a.[5)]	29.42	28.39	24.77	31.95	n. a.[5)]	n. a.[5)]	28.37
14	33.68	n. r[4)]	34.74	n. a.[5)]	n. a.[5)]	34.21	31.55	n. r[4)]	32.61	n. a.[5)]	n. a.[5)]	32.08
28	25.35	29.28	24.73	25.82	37.32	28.50	22.86	26.79	22.24	23.33	34.83	26.01
Results obtained for F_B solution (*Inoculum Control solution*, containing neither test nor reference compounds):												
DAT	***TIC [mg/L][2)]***						***Corrected TIC [mg/L][3)]***					
	Replicate					Average	Replicate					Average
	1	2	3	4	5		1	2	3	4	5	
0	n. r[4)]	n. r[4)]	0.81	n. a.[5)]	n. a.[5)]	0.81	*n. a.*[6)]	*n. a.*[6)]	*n. a.*[6)]	*n. a.*[6)]	*n. a.*[6)]	*n. a.*[6)]
2	-0.48	-0.05	-0.01	n. a.[5)]	n. a.[5)]	-0.18	*n. a.*[6)]	*n. a.*[6)]	*n. a.*[6)]	*n. a.*[6)]	*n. a.*[6)]	*n. a.*[6)]
5	1.12	1.01	1.04	n. a.[5)]	n. a.[5)]	1.05	*n. a.*[6)]	*n. a.*[6)]	*n. a.*[6)]	*n. a.*[6)]	*n. a.*[6)]	*n. a.*[6)]
7	0.48	1.18	0.86	n. a.[5)]	n. a.[5)]	0.84	*n. a.*[6)]	*n. a.*[6)]	*n. a.*[6)]	*n. a.*[6)]	*n. a.*[6)]	*n. a.*[6)]
9	1.12	0.87	0.32	n. a.[5)]	n. a.[5)]	0.77	*n. a.*[6)]	*n. a.*[6)]	*n. a.*[6)]	*n. a.*[6)]	*n. a.*[6)]	*n. a.*[6)]
14	2.80	2.45	1.14	n. a.[5)]	n. a.[5)]	2.13	*n. a.*[6)]	*n. a.*[6)]	*n. a.*[6)]	*n. a.*[6)]	*n. a.*[6)]	*n. a.*[6)]
21	2.20	1.98	0.67	n. a.[5)]	n. a.[5)]	1.62	*n. a.*[6)]	*n. a.*[6)]	*n. a.*[6)]	*n. a.*[6)]	*n. a.*[6)]	*n. a.*[6)]
28	2.36	2.26	2.58	2.48	2.78	2.49	*n. a.*[6)]	*n. a.*[6)]	*n. a.*[6)]	*n. a.*[6)]	*n. a.*[6)]	*n. a.*[6)]
Results obtained for F_I solution (*Toxicity Control solution*, containing both test and reference compounds):												
DAT	***TIC [mg/L][2)]***						***Corrected TIC [mg/L][3)]***					
	Replicate					Average	Replicate					Average
	1	2	3	4	5		1	2	3	4	5	
0	0.24	0.97	0.76	n. a.[5)]	n. a.[5)]	0.66	-0.58	0.16	-0.05	n. a.[5)]	n. a.[5)]	-0.16
5	14.99	15.08	15.92	n. a.[5)]	n. a.[5)]	15.33	13.93	14.02	14.86	n. a.[5)]	n. a.[5)]	14.27
14	19.06	21.03	14.95	n. a.[5)]	n. a.[5)]	18.36	16.93	18.90	12.82	n. a.[5)]	n. a.[5)]	16.22
28	14.04	15.30	19.82	19.01	18.01	17.24	11.55	12.81	17.33	16.52	15.52	14.74

Footnotes to the table:

1) DAT – Days After Treatment, i. e. after the test substance/control substance was applied and the incubation started;
2) TIC – Total Inorganic Carbon, value corrected by the blank value of 7M NaOH;
3) Corrected TIC – Total Inorganic Carbon corrected by the inoculum control;
4) n. r. – not reported, the results not available due to broken flask during unfreezing;
5) n. a. – value not available, not measured (replicates not set);
6) *n. a.* – not applicable.

The values presented in the table above were then converted using the equation for calculating % biodegradation. The results are given below in the table 5.11.2.4-2 (DAR Table B.8.4.3.1-2).

Table 5.1.2.4-2 (DAR Table B.8.4.3.1-2): Results of the experiment presented in form of the % biodegradation during the test period.

% biodegradation observed in :												
DAT	F_T *solution (*Test Item solution, *containing sulfoxaflor):*						F_B *solution (*Inoculum Control solution, *containing neither test nor reference compounds):*					
	Replicate					Average	Replicate					Average
	1	2	3	4	5		1	2	3	4	5	
0	0.4	-2.7	n. r[1)]	n. a.[2)]	n. a.[2)]	-1.2	*n. a.*[3)]	*n. a.*[3)]	*n. a.*[3)]	*n. a.*[3)]	*n. a.*[3)]	*n. a.*[3)]
2	-1.2	-2.7	1.4	n. a.[2)]	n. a.[2)]	-0.8	*n. a.*[3)]	*n. a.*[3)]	*n. a.*[3)]	*n. a.*[3)]	*n. a.*[3)]	*n. a.*[3)]
5	-0.9	-1.2	0.0	n. a.[2)]	n. a.[2)]	-0.7	*n. a.*[3)]	*n. a.*[3)]	*n. a.*[3)]	*n. a.*[3)]	*n. a.*[3)]	*n. a.*[3)]
7	1.2	2.2	n. r[1)]	n. a.[2)]	n. a.[2)]	1.7	*n. a.*[3)]	*n. a.*[3)]	*n. a.*[3)]	*n. a.*[3)]	*n. a.*[3)]	*n. a.*[3)]
9	1.2	1.4	0.9	n. a.[2)]	n. a.[2)]	1.2	*n. a.*[3)]	*n. a.*[3)]	*n. a.*[3)]	*n. a.*[3)]	*n. a.*[3)]	*n. a.*[3)]
14	0.1	2.5	-0.1	n. a.[2)]	n. a.[2)]	0.8	*n. a.*[3)]	*n. a.*[3)]	*n. a.*[3)]	*n. a.*[3)]	*n. a.*[3)]	*n. a.*[3)]
21	1.5	1.2	1.2	n. a.[2)]	n. a.[2)]	1.3	*n. a.*[3)]	*n. a.*[3)]	*n. a.*[3)]	*n. a.*[3)]	*n. a.*[3)]	*n. a.*[3)]
28	n. r[1)]	0.1	-3.7	n. r[1)]	-1.1	-1.6	*n. a.*[3)]	*n. a.*[3)]	*n. a.*[3)]	*n. a.*[3)]	*n. a.*[3)]	*n. a.*[3)]
% biodegradation observed in :												
DAT	F_C *solution (*Procedure Control solution, *containing reference compound- sodium benzoate):*						F_I *solution (*Toxicity Control solution, *containing both test and reference compounds):*					
	Replicate					Average	Replicate					Average
	1	2	3	4	5		1	2	3	4	5	
0	-0.4	-3.2	0.5	n. a.[2)]	n. a.[2)]	-1.0	-2.9	0.8	-0.3	n. a.[2)]	n. a.[2)]	-0.8
5	143.2	125.0	161.2	n. a.[2)]	n. a.[2)]	143.1	70.1	70.6	74.8	n. a.[2)]	n. a.[2)]	71.8
14	159.1	n. r[1)]	164.5	n. a.[2)]	n. a.[2)]	161.8	85.2	95.1	64.5	n. a.[2)]	n. a.[2)]	81.6
28	115.3	135.1	112.1	117.7	175.7	131.2	58.1	64.5	87.2	83.1	78.1	74.2

1) n. r. – not reported, the results not available due to broken flask during unfreezing;
2) value not available, not measured (replicates not set);
3) *n. a.* – not applicable.

Additionally the results reported in the table 5.11.2.4-2 (DAR Table B.8.4.3.1-2) were also presented in graphical form (figure 5.11.2.4-1; DAR Figure B.8.4.3.1-1).

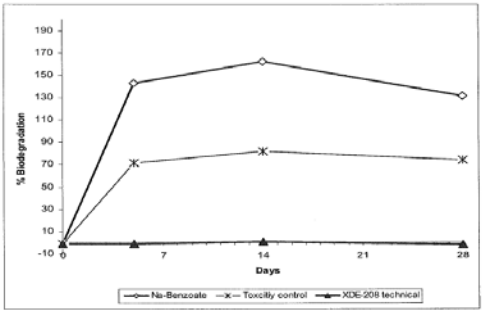


Figure 5.1.2.4-1 (DAR Figure B.8.4.3.1-1): The graphical presentation of the results of the study on biodegradation of technical sulfoxaflor.

The maximum concentration of inorganic carbon in blank samples (**F_B**) was 2.78 mg/L, therefore it was lower that the acceptable level 3 mg/L. The level of mineralization of the reference item – sodium benzoate, after 14 days of incubation was >100%. Finally, in the toxicity control solution the level of mineralization was 71.8% after 5 days, 81.6% after 14 days and 74.2% after 28 days, what indicates that the test compound – sulfoxaflor did not inhibit the process.

The level of biodegradation of sulfoxaflor during the experiment was not higher than 3%, peaking at 2.5% after 14 days. Minimal level of biodegradation was recorded in the samples incubated for 28 days.

Final conclusion:
On the basis of the results presented above it can be stated that sulfoxaflor is not ready biodegradable.

5.1.2.3 Simulation tests

The fate and behaviour of Sulfoxaflor in aquatic system – water/sediment system, was examined in two systems (both pond systems) representative for the static surface water bodies present in the UK agricultural landscape – one with coarse sediment (sand sediment), another with fine sediment (silt loam sediment) (**Laughlin L. A, Adelfinskaya Y., Balcer J. L, 2010; study report No. 080138**). The study was performed according to the OECD Guideline 308. It was evaluated and no significant deviations were noted. Therefore the study was accepted and considered reliable for the assessment of biological degradation of sulfoxaflor in the aquatic environment.

It was stated that the mechanism of dissipation of Sulfoxaflor from water phase was mixed – partly it was degraded to X11719474 and partly migrated to the sediment, where this compound underwent transformation to X11719474. It was noted that migration from a water column to the sediment was more intense in the system with fine sediment which had higher adsorption potential. It was also noted that the degradation of Sulfoxaflor was faster in the system with fine sediment, not only having higher adsorption potential but also displaying higher microbial activity.

The level of mineralization was not high; it reached a maximum level of 1.6% in fine sediment system and 0.55% in coarse sediment system. **This supports the conclusion drawn in the study on ready biodegradability of Sulfoxaflor – Sulfoxaflor is not ready biodegradable. It also indicates that X11719474 should be classified as not ready biodegradable as well.**

The level of the non-extractable residues (NER) was correlated with the texture of the sediment – it was about 4 times higher in the system with fine sediment (24.35%) than in the system with coarse sediment (6.55%). This may be also related to the described above behaviour of Sulfoxaflor in the system – the compound tended to be present mainly in water phase in the system with coarse (sand) sediment, while in the system with fine (silt loam) sediment up to 40% of its initial dose was found in the sediment relatively shortly after application (within 15 days after treatment).

The only metabolite found in both systems within the study duration was X11719474, which is also a major soil metabolite. It was formed in both systems at the maximum amount ~65-71% and displayed significantly greater persistence than the parent compound. Its distribution between water and sediment phases was very similar to that reported above for Sulfoxaflor, although, most probably due to lower adsorption potential, it showed lower affinity to sediment phase than Sulfoxaflor.

Sulfoxaflor should be regarded as not rapidly biologically degraded in the aquatic environment (water/sediment system) with the geomean whole system DT_{50} = 57.08 days and the geomean whole system DT_{90} = 189.63 days. The persistence of this compound in the sediment phase was slightly higher – the geomean sediment DT_{50} = 68.63 days and the geomean sediment DT_{90} = 244.25 days. It was noted that the degradation rates were correlated with the texture of the sediment within the test systems – it was faster in case of the system with the fine sediment (silt loam sediment).

For the degradation product X11719474 the decline phase was not reached in the experiment. It was noted however that its concentration, after reaching maximum, stabilised what may indicate that this compound is highly persistent in the aquatic environment. Therefore, as it was in case of sulfoxaflor it should be regarded as not rapidly biologically degraded in the aquatic environment (water/sediment system).

The summary of this study, as presented in the Draft Assessment Report for Sulfoxaflor, is given below.

Study 1:

Report: Laughlin L. A., Adelfinskaya Y., Balcer J. L., (2010): "Aerobic transformation of XDE-208 in Two European Aquatic Sediment Systems.". Regulatory Sciences & Government Affairs, Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, Indiana 46268-1054, USA; unpublished study report No. 080138; 18 March 2010.

Guidelines: study was carried out to comply with the following:

- OECD Guideline for the Testing of Chemicals 308 – Aerobic and Anaerobic Transformation in Aquatic Sediment Systems; Guideline adopted on 24th April 2002;
- US EPA OPPTS Guideline 835.430 – Aerobic Aquatic Metabolism; October 2008;

GLP: Yes.

Reliability of the Study: The study was evaluated using both guidelines listed above. Additionally, to verify the correctness of the kinetic evaluation of the data, following Guidance Document was consulted:

- FOCUS "Guidance document on estimating persistence and degradation kinetics from environmental fate studies on pesticides in EU registration" (FOCUS (2006) "Guidance document on estimating persistence and degradation kinetics from environmental fate studies on pesticides in EU registration" Report of the FOCUS Work Group on Degradation Kinetics, EC Document Reference SANCO/10058/2005 version 2.0, 434 pp.);

The study was evaluated, it is acceptable.

Summary:

The aim of the study was to examine the fate and behaviour of sulfoxaflor in aerobic aquatic systems by determining its route and rate of degradation.

The experiment was performed in a way to comply with the following Guidelines:

- OECD Guideline for the Testing of Chemicals 308 – Aerobic and Anaerobic Transformation in Aquatic Sediment Systems; Guideline adopted on 24th April 2002;
- US EPA OPPTS Guideline 835.430 – Aerobic Aquatic Metabolism; October 2008;

The obtained results were kinetically evaluated in line with the recommendations given in the following Guidance Document:

- FOCUS "Guidance document on estimating persistence and degradation kinetics from environmental fate studies on pesticides in EU registration" (FOCUS (2006) "Guidance document on estimating persistence and degradation kinetics from environmental fate studies on pesticides in EU registration" Report of the FOCUS Work Group on Degradation Kinetics, EC Document Reference SANCO/10058/2005 version 2.0, 434 pp.).

It was stated that no deviations from the Guidelines were observed, therefore none was reported. Having examined the study report it is confirmed that this statement was correct.

The study was performed using two water/sediment systems collected from two UK ponds, selected as representative of major agricultural areas in the UK. The detailed characteristic of water/sediment systems used in the experiment is given below in the table 5.11.2.5-1 (DAR Table B.8.4.3.2-1).

Table 5.1.2.5-1 (DAR Table B.8.4.3.2-1): The characteristic of water/sediment systems used in the experiment.

Parameters			Test system	
			M765 (Site C)	*M766 (Site A)*
General data	*Geographic location*		Chatsworth, Derbyshire, UK	Calwich, Staffordshire, UK
	Type of water body		Pond	Pond
	Collection date		15/05/2008	14/05/2008
	Pesticide use history		None used in 4 years before sampling	None used
Water phase properties	*pH*		6.7	7.8
	Redox potential [mV]	Initial	15	22
		Final	218	88
	Dissolved oxygen [mg O_2/L]	Initial	3.6	5.1
		Final	8.2	4.9
	Dissolved Organic Carbon [ppm]		6.2	6.5
	Hardness [mg equivalent $CaCO_3$/L]		25	137
	Conductivity [mmhos/cm]		0.10	0.30
Sediment properties	*Texture class - USDA*		Sand	Silt loam
	Particle size distribution - USDA	% sand	90	33
		% silt	7	61
		% clay	3	6
	Textrure class - International		Sand	Loam
	Particle size distribution - International	% sand	93	60
		% silt	4	34
		% clay	3	6
	pH		6.3	7.8
	Organic carbon content [%]		0.6	3.9
	CEC [meq/100g]		2.3	14.9
	Redox potential [mV]	Initial	-240	-356
		Final	-288	-383
	Biomass [µg/g]	Initial	75.2	463.4
		Final	58.6	51.6
	Bulk density [g/cm^3]		1.24	0.66

The sediment samples were in both cases sampled from the 0-10cm layer, as recommended by the relevant guidelines, sieved at the sampling site through 2-mm sieve and transferred to the laboratory, where the test was performed. The water samples were taken from the same water bodies as sediment samples, from the 0-45 cm layer for M765 system and 0-20cm layer for M766 system. Water samples were sieved at the sampling site through a 212-µm sieve before being transferred to the test laboratory, where they were filtered through glass wool. The samples of sediment and water were stored at T = 4^0C for less than a month before being used.

Before use the moisture content of both sediments was determined by oven drying a sub-sample of each of them. These data were then used to calculate the amount of fresh sediment and water related to it needed to obtain the recommended water:sediment ratio 3:1 – 4:1 (v:w).

The test substance used in the experiment was the radiolabelled ^{14}C-sulfoxaflor (1:1 mixture of diastereoisomers) having the chemical purity 99.7%, radiochemical purity 99.7% and specific activity of 62.0 mCi/mmole. The test substance was radiolabelled at C2 position in the pyridine ring as shown below on figure 5.11.2.5-1 (DAR Figure B.8.4.3.2-1).

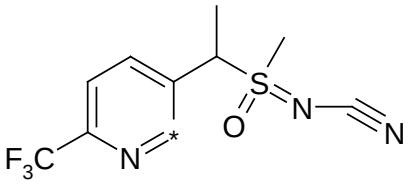


Figure 5.11.2.5-1 (DAR Figure B.8.4.3.2-1): The structural formula of the radiolabelled sulfoxaflor (XDE-208) used in the experiment. The radiolabelling position is indicated by the *.

The test substance was introduced to the test system in form of a dosing solution, prepared from the stock solution of radiolabelled ^{14}C-sulfoxaflor in acetonitrile. The nominal application rate was 0.030 mg/sulfoxaflor/L, calculated in a way presented below.

The maximum assumed application rate for the EU was 48 g/ha. Assuming the application of the entire material to 1-ha pond being 15-cm deep, the application rate to the water phase was calculated to be 0.032 mg/L.

The anticipated global maximum application rate of sulfoxaflor was 300 g/ha. Using the assumptions outlined in the US EPA guideline OPPTS 835.4300 – a pond having the area of 1ha and the depth of 100 cm, the application rate to the water phase was calculated to be 0.030 mg/L.

As the difference between these two values was minimal, the application rate of 0.030 mg/L was selected.

This application rate is acceptable as it corresponds to approximately twice the application rate of sulfoxaflor proposed in the EU-representative GAP – 24 g/ha.

The experiment lasted for 103 days. The samples were incubated in the dark, at constant temperature T = 20 ± 1^{0}C in a flow-through incubation system, presented below on figure 5.11.2.5-2 (DAR Figure B.8.4.3.2-2). Water/sediment samples were placed in 250-mL Nalgene centrifuge tubes, which were connected to the caustic trap – a glass jar filled with 2N NaOH, set for collection of produced $^{14}CO_2$. The system was constantly aerated with the moist air.

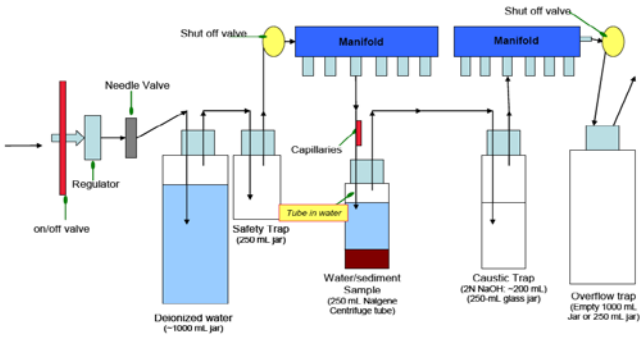


Figure 5.11.2.5-2 (DAR Figure B.8.4.3.2-2): The incubation system used in the experiment.

The amount of sediment in each tube was such to give 2.5-cm (± 0.5-cm) layer. It was determined in the following way:

250-mL centrifuge tubes, of the same kind as used in the flow-trough system, were marked at 2.5 cm using the ruler. They were then weighed and the appropriate amount of either sand sediment

(M765) or silt loam sediment (M766) was introduced to them up to the mark. The tubes with the sediment were then weighed once again. It was determined that approximately 100 g of sand sediment (M765) and approximately 80 g of silt loam sediment (M766) was needed to obtain the ~2.5-cm sediment layer. After accounting for the moisture content 72 g oven-dry sand sediment (M765) and 29 g oven-dry silt loam sediment was needed per test vessels. The amount of water added to each incubation vessel with sand sediment (related to it) was 215 mL, what gave water:sediment ratio of 3:1 (v/w). In case of silt loam water sediment system the amount of water added to each incubation vessel was 116 mL, what resulted in water:sediment ratio of 4:1(v/w). For each time point duplicate samples were prepared. The samples, after being weighed, were incubated for 7 days prior to application of the test compound under the same conditions as during the experiment, in order to allow them equilibrate to moisture and temperature.

Additionally surrogate samples were prepared, one of each type of water/sediment system per each sampling point, to monitor pH, dissolved oxygen and redox potential in water, as well as redox potential in sediment. This was done in order not to insert the instrument probes into the treated samples, what might have affected the distribution of the test compound and its degradation products in the system.

Also the determination of the biomass in the sediment at the end of the study was performed separately. This was done in 2-L glass jars containing either sand sediment (M765) or silt loam sediment (766) with pond water related to them.

First the amount of sediment needed to produce a 2.5-cm (± 0.5 cm) sediment layer in a test vessel, as well as the amount of the associated pond water required to give the appropriate water:sediment ratio, were determined. This was done in the same way as described above for the test vessels.

The prepared water/sediment samples (in two replicates for each system), attached to the air flow-trough system, were incubated in the dark at T = 20^0C for the period equal to the study duration (103 days). At study termination the samples were removed from the incubation chamber and shipped to the laboratory performing the final analysis.

The test compound was applied to the test vessels in form of the dosing solution using the positive displacement pipette. The application was onto the water surface. To each vessel the same amount of 100 µL of the dosing solution was applied. In order to obtain the same application rate of 0.030 mg sulfoxaflor/L using the constant amount of applied solution, two dosing solutions were prepared – one for sand sediment system, another for silt loam sediment system. This was due to the fact that the water:sediment ratios in these two systems, and hence the volume of water in each type of system, were different. The homogeneity and application rate of each dosing solution were controlled by taking aliquots of it during treatment. These samples were analysed using LSC technique.

At sampling times (DAT) 0, 4, 8, 15, 21, 32, 46, 61, 76, 88 and 103 duplicate treated samples and one surrogate sample of each water/sediment system and connected to them caustic traps were taken for the further analysis.

The caustic traps were analysed on the day of the sampling. For this purpose triplicate 2-mL aliquots of the trapping solution were radioassayed by LSC to determine level of mineralization. Only the traps at DAT 0 were not radioassayed, because at this time point no radioactivity was expected to be found in the traps (this in turn resulted from the assumption that at this time point no mineralization should occur).

The test vessels with water and sediment were weighed and the aqueous and sediment layers were separated by centrifugation. Aqueous layer was transferred into a labelled, weighed container using glass pipette (this was done to minimise the disturbance of the sediment layer). Then the container with collected aqueous layer was weighed and the result recorded. On the basis of the weight measurement the volume of the collected aqueous phase of each sample was determined, assuming the density of the aqueous solution being 1 g/mL. Triplicate aliquots (typically 2mL) were analysed for the radioactivity content using LSC.
The remaining amount of aqueous layer underwent pre-treatment – concentration and filtration, prior to HPLC analysis. This was done in a following way:

An aliquot of the given aqueous layer sample was loaded onto a Strata X SPE cartridge. The cartridge was then rinsed with HPLC-grade water and the residues were eluted from it with acetonitrile. A methanol:glycol (80:20) solution was then added to the eluate and the sample was evaporated on dryness under the stream of nitrogen on a Turbovap evaporator at T = 40^0C. The residues were reconstituted in acetonitrile:water (5:95) solution containing 0.1% acetic acid and filtered through a 0.2-μm filter. Aliquots of the concentrated sample were analysed by LSC to determine the recovery level. The samples were stored refrigerated until being analysed by HPLC.

To the vessel containing the sediment pellet, weighed after centrifugation and collection of aqueous phase, ~120 mL of acetonitrile:1N HCl_{aq} (90:10) solution was added. Sample was vortex –mixed to break up the pellet, placed on a horizontal shaker and shaken at low speed for 1 hour, then centrifuged for 5 minutes at 2000 rpm. The extract was decanted into a weighed labelled jar and the extraction of the pellet was repeated twice with fresh 100-mL portions of the same extracting solution. Combined extract was weighed and its three aliquots (usually 1 mL) were assayed by LSC.

The average density of extracted sample was determined by weighing aliquots of each extract. This density was used, together with measured weight of each combined extract, to determine its volume.

The extracts prior to HPLC analysis underwent the pre-treatment - concentration and filtration. This was done in a following way:

An aliquot of the given extract was neutralized to pH 6-7 with NaOH, centrifuged and the resulting solution was decanted into a clean vial. The precipitate was rinsed with acetonitrile, centrifuged and the resulting solution pooled with the first decant. The pooled solution was then concentrated to less than 10 mL dryness under the stream of nitrogen on a Turbovap evaporator at T = 40^0C, reconstituted with ~10 mL HPLC-grade water and 100 μL aliquots (three) analysed using LSC. The remaining solution was loaded onto a pre-conditioned Strata X SPE cartridge. The cartridge was then rinsed with HPLC-grade water and the residues were eluted from it with acetonitrile. A methanol:glycol (80:20) solution was then added to the eluate and the sample was evaporated on dryness under the stream of nitrogen on a Turbovap evaporator at T = 40^0C. The residues were reconstituted in acetonitrile:water (5:95) solution containing 0.1% acetic acid and filtered through a 0.2-μm filter. Aliquots of the concentrated sample were analysed by LSC to determine the recovery level. The samples were stored refrigerated until being analysed by HPLC.

The extracted sediment pellets were allowed to air dry in a hood for at least one week before being analysed for the NER (non-extractable residues) content. In order to do this three

~0.5-g. subsamples of each previously extracted, air-dried sediment were combusted using a biological oxidizer. The generated $^{14}CO_2$ was collected in scintillation cocktail and analysed using LSC.

The NER were further characterised in a following way:

Subsamples (~5 g.) of previously extracted, air-dried sediment were transferred into centrifuge tubes and extracted with 25 mL of 0.5M NaOH for 24 hours, on a mechanical shaker, at room temperature. Samples were centrifuged, supernatants collected and pellets were briefly extracted with fresh 25-mL portion of 0.5M NaOH. The extracts, after centrifugation were pooled with the original extracts and the pellets were rinsed with 25 mL of deionised water, which, after centrifugation were combined with the original extracts.

Supernatants, after acidification, were allowed to stand at room temperature overnight, then they were centrifuged and the resulting supernatants were transferred to a 100-mL volumetric flask and filled to the mark with deionised water. This fraction, further called fulvic acid fraction, was analysed by LSC.

The precipitate, further called humic acid fraction, was redissolved in 25 mL of NaOH and its triplicate aliquots analysed using LSC technique.

Radioactivity remaining in extracted pellet after alkaline extraction (determined by subtraction of radioactivity in fulvic-acid and humic-acid fractions from the total NER) was defined as humin-associated fraction.

The LSC analysis of the samples was performed immediately after their preparation.
To each sample before counting a scintillation cocktail was added. Samples were generally counted for 5 minutes, however for samples with low expected level of radioactivity the counting time was extended to 10 minutes or longer, if needed. The reference ^{14}C calibration standards were used on the day of analysis of the samples to verify the performance of the LSC apparatus. The LOD and LOQ levels for this analysis were determined using the method of Currie.

The qualitative and quantitative analysis of the samples was performed by RP-HPLC method. The analytical system was equipped with Ascentis Express C18 (150x4.6 mm; 2.7 μm) chromatographic column and working in a gradient mode. This system was equipped with two detectors:

- an UV-Vis detector set to one wavelength – λ = 254 nm, to determine the retention times of the non-radiolabelled standards (qualitative analysis);
- a radioactive flow-through detector (RAM) used to quantitate the relative percent of radioactivity in chromatographed solution (quantitative analysis);

The chromatographic analysis of each sample, performed at ambient temperature of the column, lasted for 67 minutes; the flow rate was set to 1.0 mL/min. The mobile phase consisted of two solvents:

- Solvent A: 0.1% acetic acid in water;
- Solvent B: 0.1% acetic acid in acetonitrile.

The gradient mode used in chromatographic analysis is presented below in the table 5.1.2.5-2 (DAR Table B.8.4.3.2-2).

Table 5.1.2.5-2 (DAR Table B.8.4.3.2-2): The gradient mode used in RP-HPLC analysis.

Event	Time [min]	Solvent ratio	
		Solvent A [%]	*Solvent B [%]*
Initial conditions	0.0	100	0
Shallow gradient	40.0	75	25
Hold	45.0	75	25
Steep gradient	47.0	5	95
Organic hold	55.0	5	95
Return to initial conditions	57.0	100	0
Equilibration	67.0	100	0

The LOD and LOQ levels for the LSC method were calculated using the method of Currie. However, the same method, due to the complexity of calculations could not be used in HPLC analysis. Therefore another way of determination of LOD and LOQ values was used.

Additionally the confirmatory analysis for the identification of the compounds detected in the study was performed for some samples using LC/MS/MS technique.

The LC/MS/MS system was Thermo Accela HPLC, coupled with Thermo LTQ FT Ultra mass spectrometer, system equipped with Supelco Ascentis C_{18} (150x4.6 mm; 2.7 μm) chromatographic column and working in a gradient mode. Additionally a Berthold radioactivity monitor (RAM) was used to assist in location of the chromatographic peaks. The HPLC split flow ratio MS: RAM was approx. 20:80.

The chromatographic analysis of each sample, performed at ambient temperature of the column, lasted for 80 minutes; the flow rate was set to 1.0 mL/min. The mobile phase consisted of two solvents:

- Solvent A: 0.1% formic acid in water;
- Solvent B: 0.1% formic acid in acetonitrile.

The gradient mode used in chromatographic analysis is presented below in the table 5.1.2.5-3 (DAR Table B.8.4.3.2-3).

Table 5.1.2.5-3 (DAR Table B.8.4.3.2-3): The gradient mode used in LC/MS/MS analysis.

Time [min]	Solvent ratio	
	Solvent A [%]	*Solvent B [%]*
0:00	95	5
10:00	95	5
35:00	80	20
40:00	80	20
65:00	10	90
70:00	10	90
75:00	95	5
80:00	95	5

The MS/MS detection system worked in ESI positive mode.

Results and their discussion:
For the LSC method the LOD was 10 dmp (decays per minute) above the background, while LOQ = 40 dmp (decays per minute) above the background.

For the HPLC method LOD = 1% AR, while LOQ was set to 3LOD – 3% AR.

The results of the measurements of physical conditions in the test systems (pH, dissolved oxygen, redox potential) are presented below in the table 5.11.2.5-4 (DAR Table B.8.4.3.2-4):

Table 5.1.2.5-4 (DAR Table B.8.4.3.2-4): The physico-chemical parameters of the test systems.

Test system	Sampling point - DAT	Physico-chemical parameters of water phase:				Physico-chemical parameters of sediment phase	
		pH	*Dissolved O_2 [ppm]*	*Redox potential – E_h [mV]*		*Redox potential – E_h [mV]*	
				actual[1)]	E_h7[2)]	actual[1)]	E_h7[2)]
Sand sediment system (M765)	***0***	7.07	3.6	19	15	-236	-240
	4	7.42	6.1	114	89	-273	-298
	8	7.68	6.5	83	43	-285	-325
	15	6.39	7.3	187	223	-275	-239
	21	6.88	3.0	116	123	-289	-282
	32	6.56	7.4	170	196	-269	-243
	46	5.98	8.8	218	278	-273	-213
	61	6.31	8.2	239	280	-278	-237
	76	7.23	7.9	228	214	-249	-263
	88	7.23	7.7	217	203	-252	-266
	103	7.53	8.2	249	218	-257	-288
Silt loam sediment system (M766)	***0***	8.15	5.1	90	22	-288	-356
	4	8.17	5.9	117	48	-337	-406
	8	7.36	2.0	78	57	-291	-312
	15	7.61	4.2	142	106	-291	-327
	21	7.75	4.5	140	96	-358	-402
	32	8.48	7.1	137	49	-299	-387
	46	8.21	8.2	168	96	-259	-331
	61	8.02	6.9	126	66	-287	-347
	76	8.17	6.6	117	48	-281	-350
	88	7.41	2.2	106	82	-279	-303
	103	7.76	4.9	133	88	-338	-383

1) Measured redox potential;
2) Redox potential corrected to that at pH = 7; following equation was used $E_h7 = E_h + \Delta E_h$, where ΔE_h = -59.2 mV *(pH-7).

The results presented above demonstrate that the physicochemical conditions in the system were relatively stable throughout the whole study and that the aerobic conditions in the water phase were maintained. However, the results of the measurements of the redox potential of the sediment indicate that in this phase conditions were anaerobic.

The results of the determination of the sediment biomass at the beginning and at the end of the study are presented in the table 5.11.2.5-1 (DAR Table B.8.4.3.2-1). On their basis it can be stated that while in sand sediment system (M765) the decrease of the biomass at the end of the study was ~22% (from 75.2 μg/g to 58.6 μg/g), it was significant in case of the second system (silt loam system – M766) – almost 89% drop in the biomass content was recorded (from 463.4 μg/g to 51.6 μg/g).

The verification of the analytical method used in the experiment gave following results:

- the extraction efficiency experiment, performed prior to the sample treatment demonstrated that the amount of AR recovered in three extractions was above 94%, what indicated the suitability of the extraction procedure; also the level of NER up to DAT 30 indicated that the extraction procedure, adjusted for the sediment weight, was acceptable;
- verification of the HPLC procedures determined by the comparison of radioactivity eluted from the column and that determined directly by LSC showed that the recovery levels were 90-110%, therefore the chromatographic procedure was acceptable;

- finally the verification of method repeatability performed for the aqueous samples and organic extract demonstrated good repeatability of the results, what in turn indicated that the analytical method and instrumentation were acceptable.

The distribution of radioactivity in the water/sediment systems is presented below, separately for sand sediment system (M765) and silt loam sediment system (M766), in tables 5.11.2.5-5 (DAR Table B.8.4.3.2-5) and 5.11.2.5-6 (DAR Table B.8.4.3.2-6). The average values were calculated.

Table 5.1.2.5-5 (DAR Table B.8.4.3.2-5): The mass balance, expressed as % AR, in sand sediment system (M765).

DAT	Replicate	AR [%] recovered as:				Total AR recovered [%]
		in aqueous layer	*extracted from sediment (organic extract)*	*CO_2 (in caustic traps)*	*NER*	
0	1	95.6	0.6	Not available	0.0	96.2
	2	100.6	0.4	Not available	0.0	101.0
	average	*98.1*	*0.5*	----	*0.0*	*98.6*
4	1	94.9	5.7	0.0	0.1	100.7
	2	95.1	4.3	0.0	0.0	99.5
	average	*95.0*	*5.0*	*0.0*	*0.05*	*100.1*
8	1	94.0	5.5	0.0	0.1	99.6
	2	92.3	8.7	0.0	0.1	101.2
	average	*93.15*	*7.1*	*0.0*	*0.1*	*100.4*
15	1	84.9	13.0	0.0	0.6	98.5
	2	84.3	15.5	0.1	0.9	100.7
	average	*84.6*	*14.25*	*0.05*	*0.75*	*99.6*
21	1	79.4	16.9	0.0	1.2	97.6
	2	81.4	16.8	0.0	1.2	99.5
	average	*80.4*	*16.85*	*0.0*	*1.2*	*98.55*
32	1	79.1	18.2	0.1	2.0	99.5
	2	77.6	18.0	0.1	1.8	97.6
	average	*78.35*	*18.1*	*0.1*	*1.9*	*98.55*
46	1	77.9	19.3	0.2	3.7	101.0
	2	78.2	19.5	0.1	3.5	101.3
	average	*78.05*	*19.4*	*0.15*	*3.6*	*101.15*
61	1	74.8	19.0	0.3	6.1	100.2
	2	77.3	18.9	0.2	4.1	100.6
	average	*76.05*	*18.95*	*0.25*	*5.1*	*100.4*
76	1	75.2	19.4	0.3	4.7	99.5
	2	81.2	19.7	0.2	4.2	105.4
	average	*78.*	*19.55*	*0.25*	*4.45*	*102.45*
88	1	75.4	20.9	0.3	4.1	100.7
	2	72.5	19.8	0.3	6.3	98.9
	average	*73.95*	*20.35*	*0.3*	*5.2*	*99.8*
103	1	70.7	21.3	0.5	6.4	98.9
	2	72.0	19.5	0.6	6.7	98.8
	average	*71.35*	*20.4*	*0.55*	*6.55*	*98.85*

Table 5.1.2.5-6 (DAR Table B.8.4.3.2-6): The mass balance, expressed as % AR, in silt loam sediment system (M766).

DAT	Replicate	AR [%] recovered as:				Total AR recovered [%]
		in aqueous layer	*extracted from sediment (organic extract)*	*CO_2 (in caustic traps)*	*NER*	
0	1	98.7	1.4	Not available	0.1	100.2
	2	99.9	0.6	Not available	0.0	100.5
	average	*99.3*	*1.0*	----	*0.05*	*100.35*
4	1	67.1	31.7	0.1	1.6	100.5
	2	69.4	28.9	0.0	1.4	99.7
	average	*68.25*	*30.3*	*0.05*	*1.5*	*100.1*
8	1	59.3	37.7	0.0	3.0	100.0
	2	58.9	38.2	0.0	2.9	100.0
	average	*59.1*	*37.95*	*0.0*	*2.95*	*100.0*
15	1	47.7	44.1	0.0	6.2	98.0
	2	47.5	44.3	0.1	6.4	98.3
	average	*47.6*	*44.2*	*0.05*	*6.3*	*98.15*
21	1	44.5	44.0	0.0	8.1	96.6
	2	44.7	45.6	0.0	7.6	97.9
	average	*44.6*	*44.8*	*0.0*	*7.85*	*97.25*
32	1	41.1	46.7	0.1	9.8	97.8
	2	41.6	45.4	0.2	10.0	97.1
	average	*41.35*	*46.05*	*0.15*	*9.9*	*97.45*
46	1	37.6	46.3	0.1	14.4	98.5
	2	39.1	45.2	0.6	13.2	98.1
	average	*38.5*	*45.75*	*0.35*	*13.8*	*98.3*
61	1	43.1	41.8	0.9	16.2	102.0
	2	41.0	42.6	0.7	15.0	99.2
	average	*42.05*	*42.2*	*0.8*	*15.6*	*100.6*
76	1	37.6	42.8	0.6	21.3	102.3
	2	37.2	43.6	0.5	21.4	102.7
	average	*37.4*	*43.2*	*0.55*	*21.35*	*102.5*
88	1	39.9	40.5	1.7	20.8	103.0
	2	38.0	41.5	1.5	20.4	101.4
	average	*38.95*	*41.0*	*1.6*	*20.6*	*102.2*
103	1	38.7	37.8	1.5	23.0	101.1
	2	35.6	39.5	0.7	25.7	101.5
	average	*37.15*	*38.65*	*1.1*	*24.35*	*101.3*

The level of recovery of the applied radioactivity was high in both systems: on average it was 99.9% (96.2 – 105.4%) in sand sediment system (M765) and 99.8% (96.6 – 103.0%) in silt loam sediment system (M766). It was also noted that there was no systematic decrease in the recovery level towards the end of the study, what indicated that no volatile compounds other than $^{14}CO_2$ collected in caustic traps were formed.

The level of mineralization in both systems was generally low, not surpassing 2% in both systems. It was noted that mineralization was slightly higher in silt loam sediment system – max. 1.7% (recorded at DAT 88), while in sand system the maximum amount of produced $^{14}CO_2$ was only 0.6% (recorded at DAT 103). These results confirm the results of the study on the ready biodegradability – sulfoxaflor is not readily biodegradable in the aquatic environment.

In sand sediment system (M765), where the sediment was coarse, with low organic carbon content (0.6%) and CEC (2.3 meq/100g), the applied radioactivity was found mainly in water phase - ~71% at the end of the study, while the radioactivity extracted from the sediment constituted only ~21% of that applied. Additionally it was stated that the amount of radioactivity extracted from the

sediment, together with NER level determined in the sediment, increased gradually. In contrast, in silt loam sediment system (M766) with fine sediment, containing 3.9% OC and having much higher CEC (14.9 meq/100g) the level of radioactivity extracted from the sediment was much higher – it peaked at 46.06% (average value) at DAT 46, but from DAT 8 onwards it stabilised on the level 37-46% AR. Additionally this increase, unlike in the sand sediment system, was rapid and already at DAT 4 (second sampling time point) it reached the level of ~30% AR (28.9 – 31.7% AR). It was also noted that in silt loam sediment the level of NER at later time points was much higher than in sand sediment system.

Finally it shall be pointed out that the OC and CEC values indicate that fine sediment, such as silt loam sediment, may have higher adsorption potential that the coarse sediment, where sand dominates.

As a result it can be stated that in the natural water bodies with fine bottom sediment, such as silt loam, reach in organic matter and having high sorption capacity, migration to the sediment should be regarded as an important dissipation mechanism of sulfoxaflor and its metabolites from water phase. In case of the natural water bodies with coarse bottom sediment, with low organic carbon content and adsorption capacity, sulfoxaflor and its metabolites are expected to be present mainly in the water phase and undergo transformation, mainly through biological degradation, there.

The level of NER varied, depending on the system. It was much lower in the sand sediment system – max. 6.7% AR at DAT 103 (end of the study) than in the silt loam sediment system – max. 25.7% AR at DAT 103 (end of the study). This may indicate that the formation of NER may be a significant route of dissipation of sulfoxaflor and its metabolites in fine sediments, reach in organic matter. The results of the further examination of the nature of NER demonstrated that radioactivity was bound predominantly to the humin fraction (57 -78% of NER fraction) and to lower amount to the fulvic acids fraction (~20% of NER fraction). Only small amount of NER fraction was bound to humic acids fraction.

The results of the further characterisation of the non-extractable residues (NER) are presented below in the table 5.11.2.5-7 (DAR Table B.8.4.3.2-7). The data are presented for both systems, for the sample collected on DAT 61. The averages were calculated.

Table 5.1.2.5-7 (DAR Table B.8.4.3.2-7): The results of the characterisation of NER (results for the samples collected on DAT 61).

System	Replicate	NER [% AR]	Non extracted radioactivity in fraction [%]			NER, as %AR, in fraction:		
			Fulvic	*Humic*	*Humin*	*Fulvic*	*Humic*	*Humin*
Sand sediment system (M765)	1	6.1	21.6	8.6	69.8	1.3	0.5	4.3
	2	4.1	28.6	13.7	57.7	1.2	0.6	2.4
	average	*5.1*	*25.1*	*11.15*	*63.75*	*1.25*	*0.55*	*3.3*
Silt loam sediment system (M766)	1	16.2	20.0	2.0	78.0	3.2	0.3	12.6
	2	15.0	22.1	2.2	75.7	3.3	0.3	11.4
	average	*15.6*	*21.05*	*2.1*	*76.85*	*3.25*	*0.3*	*12.0*

The further examination of the radioactivity in aqueous layer and extractable from the sediment demonstrated that only two compounds could be identified in both systems – sulfoxaflor and X11719474. Additionally a small fraction of the non-identified compounds, further called “other” was detected. Their concentrations in the system, together with the distribution among water and sediment phases are presented below, in the tables 5.11.2.5-8 (DAR Table B.8.4.3.2-8) for sand

sediment system (M765) and 5.11.2.5-8 (DAR Table B.8.4.3.2-9) for silt loam sediment system (M766). It was noted that while for sulfoxaflor and X11719474 the distribution in water and sediment phases was reported together with their total concentration, in case of the "other" fraction only the total concentration was given. The average concentrations were calculated.

Table 5.1.2.5-8 (DAR Table B.8.4.3.2-8): Concentrations (total, in water phase and in sediment phase) of sulfoxaflor, X11719474 and the "other" fraction, expressed as % AR, in sand sediment system (M765).

DAT	Replicate	Concentration, in %AR, of:						
		Sulfoxaflor			*X11719474*			*Other*
		Total	Water	Sediment	Total	Water	Sediment	
0	1	96.2	95.6	0.6	0.0	0.0	0.0	0.0
	2	100.4	100.0	0.4	0.0	0.0	0.0	0.6
	average	*98.3*	*97.8*	*0.5*	*0.0*	*0.0*	*0.0*	*0.3*
4	1	98.7	93.1	5.6	0.3	0.3	0.0	1.5
	2	97.7	93.4	4.3	0.0	0.0	0.0	1.7
	average	*98.2*	*93.25*	*4.95*	*0.15*	*0.15*	*0.0*	*1.6*
8	1	96.7	91.2	5.5	1.7	1.7	0.0	1.1
	2	98.6	90.2	8.5	1.8	1.7	0.1	0.5
	average	*97.65*	*90.7*	*7.0*	*1.75*	*1.7*	*0.05*	*0.8*
15	1	93.3	80.8	12.5	3.2	3.0	0.2	1.4
	2	89.9	75.4	14.5	8.2	7.3	0.9	1.7
	average	*91.6*	*78.1*	*13.5*	*5.7*	*5.15*	*0.55*	*1.55*
21	1	85.3	70.1	15.2	9.8	8.4	1.4	1.1
	2	91.6	75.7	15.9	6.0	5.1	0.9	0.6
	average	*88.45*	*79.2*	*15.55*	*7.9*	*6.75*	*1.15*	*0.85*
32	1	79.0	63.3	15.7	16.5	14.2	2.4	1.8
	2	81.9	65.6	16.2	11.9	10.3	1.6	1.8
	average	*80.45*	*64.45*	*15.95*	*14.2*	*12.25*	*2.0*	*1.8*
46	1	84.9	67.8	17.1	11.2	9.2	1.9	1.1
	2	79.8	63.7	16.1	16.2	13.1	3.1	1.7
	average	*82.35*	*65.75*	*16.6*	*13.7*	*11.15*	*2.5*	*1.4*
61	1	56.3	44.1	12.2	37.0	30.4	6.6	0.5
	2	78.6	62.5	16.1	17.0	14.3	2.6	0.6
	average	*67.45*	*53.3*	*14.15*	*27.0*	*22.35*	*4.6*	*0.55*
76	1	48.5	36.6	11.9	45.0	37.9	7.1	1.0
	2	29.2	22.5	6.7	70.9	58.2	12.7	0.8
	average	*38.85*	*29.55*	*9.3*	*57.95*	*48.05*	*9.9*	*0.9*
88	1	44.4	33.7	10.6	51.2	41.5	9.8	0.7
	2	67.0	52.4	14.6	24.7	19.8	4.9	0.6
	average	*55.7*	*43.05*	*12.6*	*37.95*	*30.65*	*7.35*	*0.65*
103	1	47.2	36.3	10.9	43.8	34.0	9.8	1.0
	2	45.0	33.6	11.4	44.8	37.2	7.7	1.7
	average	*46.1*	*34.95*	*11.15*	*44.3*	*35.6*	*8.75*	*1.35*

The concentration of sulfoxaflor in the system steadily decreased from 96.2-100.4% AR to 45.0-47.2 % AR. It was noted that the compound was present mainly in water phase, where its concentration declined from ~95 – 100% AR to 33.6 – 36.3% AR at DAT 103 (end of the study). It was also noted that the dissipation of sulfoxaflor from water phase can be attributed mainly to the degradation of this compound, while migration to sediment played only minor part in this process. The concentration of sulfoxaflor in sediment slowly increased to reach the level of 16.1 – 17.1% AR at DAT 46, afterwards it decreased to reach the level of 10.9 – 11.4% AR at the end of the study (DAT 103). Therefore it can be stated that the dissipation of sulfoxaflor from water phase had mixed character, although degradation predominated.

The only degradation product detected in the system was X11719474, which reached its maximum concentration of 45 – 70.9 % AR on DAT 76. From that time point onwards the concentration of this compound in the whole system stabilised at the level of ~45 – 50% AR. As it was in case of

sulfoxaflor, X11719474 was observed mainly in water phase, where it peaked at 38 – 58% AR on DAT 76, then its concentration stabilised at the level of 35 – 41% AR with no distinguishable decline phase (the same observation was made for the whole system). In the sediment phase the slow increase of the concentration of this compound was observed until DAT 76, when it peaked at 7.1 – 12.7% AR. Then the concentration of X11719474 in sediment stabilised at 5 – 10% AR with no visible tendency to decline. The statement that X11719474 was the sole identifiable degradation product of sulfoxaflor in this system was confirmed by the results of LC/MS/MS analysis.

The "other" fraction was recorded at rather stable level of 0.5 – 1.8% AR with no distinguishable formation/decline pattern.

Table 5.1.2.5-9 (DAR Table B.8.4.3.2-9): Concentrations (total, in water phase and in sediment phase) of sulfoxaflor, X11719474 and the "other" fraction, expressed as % AR, in silt loam sediment system (M766).

DAT	Replicate	Concentration, in %AR, of:						
		Sulfoxaflor			*X11719474*			*Other*
		Total	Water	Sediment	Total	Water	Sediment	
0	1	99.3	97.9	1.4	0.0	0.0	0.0	0.9
	2	100.0	99.6	0.5	0.0	0.0	0.0	0.4
	average	*99.65*	*98.75*	*0.95*	*0.0*	*0.0*	*0.0*	*0.65*
4	1	96.2	65.3	30.9	2.0	1.5	0.4	0.7
	2	97.8	69.4	28.4	0.2	0.0	0.2	0.3
	average	*97.0*	*67.35*	*29.65*	*1.1*	*0.75*	*0.3*	*0.5*
8	1	92.8	56.4	36.3	2.4	1.8	0.6	1.8
	2	93.8	56.9	36.9	1.9	0.9	0.9	1.5
	average	*93.3*	*56.65*	*36.6*	*2.15*	*1.35*	*0.75*	*1.65*
15	1	84.6	44.1	40.5	5.9	3.1	2.8	1.3
	2	81.6	41.8	39.8	7.8	4.4	3.5	2.4
	average	*83.1*	*42.95*	*40.15*	*6.85*	*3.75*	*3.15*	*1.85*
21	1	79.0	39.8	39.2	8.3	3.7	4.6	1.2
	2	71.8	33.5	38.3	17.4	10.7	6.6	1.1
	average	*75.4*	*36.65*	*38.75*	*12.85*	*7.2*	*5.6*	*1.15*
32	1	59.1	25.8	33.4	27.6	14.5	13.1	1.1
	2	53.8	23.0	30.8	32.3	18.3	13.9	0.9
	average	*56.45*	*24.4*	*32.1*	*29.95*	*16.4*	*13.5*	*1.0*
46	1	57.7	24.8	32.9	25.3	12.5	12.8	0.9
	2	37.0	12.9	24.1	46.8	25.9	20.9	0.5
	average	*47.35*	*18.85*	*28.5*	*36.05*	*19.2*	*16.85*	*0.7*
61	1	24.8	8.2	16.6	59.4	34.6	24.8	0.7
	2	23.5	7.0	16.5	59.1	33.3	25.8	0.9
	average	*24.15*	*7.6*	*16.55*	*59.25*	*33.95*	*25.3*	*0.8*
76	1	28.1	7.9	20.1	51.7	29.4	22.3	0.7
	2	33.3	11.8	21.5	47.1	25.3	21.8	0.5
	average	*30.7*	*9.85*	*20.8*	*49.4*	*27.35*	*22.05*	*0.6*
88	1	13.1	3.5	9.6	66.0	35.9	30.1	1.4
	2	13.7	3.4	10.3	65.1	34.6	30.5	0.7
	average	*13.4*	*3.45*	*9.95*	*65.55*	*35.25*	*30.3*	*1.05*
103	1	10.5	2.3	8.2	64.3	36.1	28.3	1.7
	2	22.6	6.3	16.3	50.8	28.6	22.2	1.7
	average	*16.55*	*4.3*	*12.25*	*57.55*	*32.35*	*25.25*	*1.7*

In this system steady decrease of the concentration of sulfoxaflor was observed, from 99.3 – 100% AR at the beginning of the study to 10.5 – 22.6% AR at its end (DAT 103). It shall be noted that sulfoxaflor in the system with fine sediment was degraded more rapidly than in the system with coarse sediment, what indicates the level of sulfoxaflor remaining at the end of the study (aver. 16.55% AR in the system with fine sediment versus aver. 46.1% AR in the system with coarse sediment). It was also noted that transformation into NER played a significant part in the degradation of sulfoxaflor in this system (23.0 – 25.7% AR in this system was in form of NER at

the end of the study).
Migration to the sediment was a significant dissipation mechanism of sulfoxaflor from the water phase – already at DAT 4 28.4 – 30.9% AR in form of sulfoxaflor was detected in sediment. This value slightly increased at the next time points to reach the maximum on DAT 15: 39.8 – 40.5% AR. From that time point onwards the concentration of sulfoxaflor in sediment decreased gradually, although it was slightly higher than the concentration of this compound in water phase.

The only degradation product identified in the system was X11719474. It reached the maximum concentration in the system 65.1 – 66.0% AR on DAT 88, which was the penultimate sampling point. As on the next time point – DAT 103 (which was the last time point in this study) the concentration decreased only slightly – to the level of 50.8 – 64.3% AR, it cannot be stated that the decline phase started.

The distribution of X11719474 in the system was very similar to that recorded for sulfoxaflor, with the approximate ratio 1:1 and concentrations in water phase slightly higher than in the sediment phase. As the concentrations of X11719474 in water and sediment phases are seemingly correlated with the corresponding concentrations of sulfoxaflor (i. e. recorded at the same time points), following things can be stated:

- the dissipation of sulfoxaflor from water phase after the concentration of this compound in sediment reaches its maximum is still of the mixed nature – partly it is degradation to X11719474 and partly migration to the sediment, where it is transformed to X11719474;
- for X11719474 there exists a sort of equilibrium as to its distribution in the system – the approximate distribution ratio water:sediment is 1:1 with an observed tendency to the concentration increase in water phase with a general increase of the whole system concentration of X11719474;
- possible interchange between the phases in case of both sulfoxaflor and X11719474 cannot be excluded.

These statements will have an influence on the selection of the kinetic endpoints derived from the study for the SW modelling.

On the basis of the results presented above in the tables 5.11.2.5-5 – 5.11.2.5-9 (DAR Tables B.8.4.3.2-5 – B.8.4.3.2-9) the following degradation scheme for sulfoxaflor in aerobic water/sediment systems was proposed (figure 5.11.2.5-3; DAR Figure B.8.4.3.2-3):

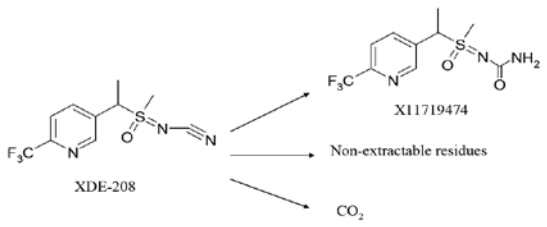


Figure 5.11.2.5-3 (DAR Figure B.8.4.3.2-3): The proposed degradation scheme for sulfoxaflor in aerobic water/sediment systems.

The data for sulfoxaflor presented in the tables 5.11.2.5-8 (DAR Table B.8.4.3.2-8) and 5.11.2.5-9 (DAR Table B.8.4.3.2-9) were kinetically evaluated in line with the recommendations given in the FOCUS Kinetics Guidance Document (FOCUS, 2006). The analysis was performed for the whole

system data, as well as for these obtained in water and sediment phases. Kinetic fitting of the data was performed using KinGUI ver. 1.1 (Bayer CropScience) modelling tool. Having analysed the data it was stated that the kinetic analysis was performed the level P-I only and further analysis at the level P-II, using a two-compartmental approach, was not performed. It was also stated that the kinetic analysis of the data for the metabolite X11719474 was not performed as for this compound the decline phase was not reached.

The data used in the kinetic evaluation were the same as reported either in the table 5.11.2.5-8 (DAR Table B.8.4.3.2-8) for sand sediment system (M765) or in the table 5.11.2.5-9 (DAR Table B.8.4.3.2-9) for silt loam sediment system (M766). No adjustments were necessary as no concentrations below the LOD were observed. For each time point the concentrations for both replicates were used (n. b.: the average values were not used; these were calculated in the review phase also).

As recommended by FOCUS Kinetics Guidance Document two kinetic models were used first – SFO and FOMC, in order to determine the best-fit kinetics. When necessary the second bi-phasic model – DFOP was also tested. The results are presented below, separately for each water/sediment system.

a) The results of the kinetic evaluation of the data obtained in sand sediment system (M765):

Kinetic evaluation of the whole system (water/sediment) data:

Two models were used in the kinetic examination of the data for sulfoxaflor obtained in the whole system (water/sediment): SFO and FOMC. The results of this examination are presented below, in graphical form on figure 5.11.2.5-4 (DAR FigureB.8.4.3.2-4) and in numerical form in the table 5.11.2.5-10 (DAR Table B.8.4.3.2-10).

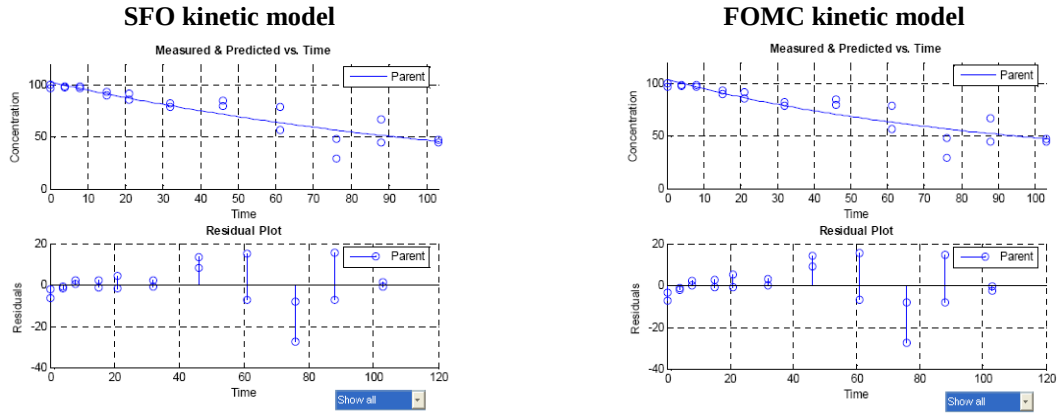


Figure 5.1.2.5-4 (DAR Figure B.8.4.3.2-4): The graphical results of the kinetic examination of the whole system data for sulfoxaflor obtained in the sand sediment system (M765).

Table 5.1.2.5-10 (DAR Table B.8.4.3.2-10): The numerical results of the kinetic examination of the whole system data for sulfoxaflor obtained in the sand sediment system (M765).

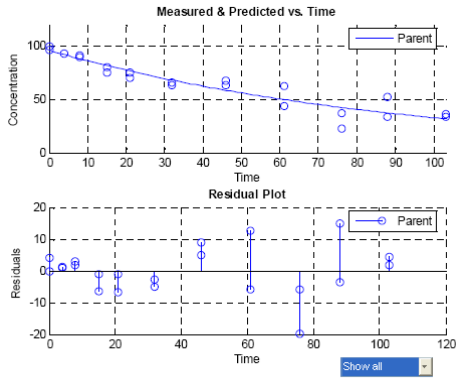
Kinetic model	Model parameters	Statistical evaluation of the parameter					Statistical evaluation of the fit		Kinetic endpoints (best-fit values)	
		Value	*Error*	*Confidence intervals*		*Prob. > t*	χ^2 *% error*	R^2	DT_{50} *[days]*	DT_{90} *[days]*
				Lower	Upper					
SFO	M_0	102.6	3.5	94.5	109.9	n. d.	7.0	0.8295	88	294
	k	0.0078	0.0009	0.0060	0.0097	1.3 E-8				
FOMC	M_0	103.6	4.8	93.6	113.6	n. d.	7.6	0.8208	89	445
	α	2.2	5.6	-9.6	14.0	0.3495				
	β	243	716	<-1000	> 1000	0.3692				

The degradation curves obtained with SFO and FOMC models are very similar, the same can be said with regard to the residuals. Also the statistical evaluation of both fits returned the similar results, although the parameters were slightly better in case of SFO model. Additionally it was stated that the model parameters for FOMC fit - α and β were statistically not reliable – the CI values for them passed through zero. For this reason the SFO model should be considered as that returning the reliable persistence and modelling endpoints. It was noted that the submission used the rounding procedure. Therefore it was decided to recalculate the DT_{50} and DT_{90} values using the degradation rate constant *k* reported in the table 5.11.2.5-10 (DAR Table B.8.4.3.2-10). The resulting kinetic endpoints, reported with two digits after the decimal point, are following: DT_{50} = 88.86 days, DT_{90} = 295.20 days.

Kinetic evaluation of the water phase data:

Two models were used in the kinetic examination of the data for sulfoxaflor obtained in the water phase: SFO and FOMC. The results of this examination are presented below, in graphical form on figure 5.11.2.5-5 (DAR Figure B.8.4.3.2-5) and in numerical form in the table 5.11.2.5-11 (DAR Table B.8.4.3.2-11). The submission adjusted the concentration at DAT 0 by adding the amount of sulfoxaflor found at that date in sediment to that reported for the water phase (this was done for both replicates).

SFO kinetic model



FOMC kinetic model

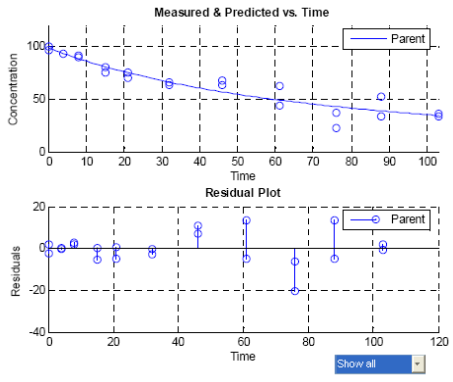


Figure 5.1.2.5.-5 (DAR FigureB.8.4.3.2-5): The graphical results of the kinetic examination of the water phase data for sulfoxaflor obtained in the sand sediment system (M765).

Table 5.1.2.5-11 (DAR Table B.8.4.3.2-11): The numerical results of the kinetic examination of the water phase data for sulfoxaflor obtained in the sand sediment system (M765).

Kinetic model	Model parameters	Statistical evaluation of the parameter					Statistical evaluation of the fit		Kinetic endpoints (best-fit values)	
		Value	*Error*	*Confidence intervals*		*Prob. > t*	χ^2 *% error*	R^2	DT_{50} *[days]*	DT_{90} *[days]*
				Lower	Upper					
SFO	M_0	96.2	3.0	90.1	102.4	n. d.	6.7	0.9013	64	214
	k	0.0108	0.0009	0.0088	0.0127	1.3 E-10				
FOMC	M_0	98.6	4.0	90.2	107.0	n. d.	6.8	0.9036	61	347
	α	1.69	1.75	-1.96	5.35	0.1721				
	β	120	158	-210	450	0.2279				

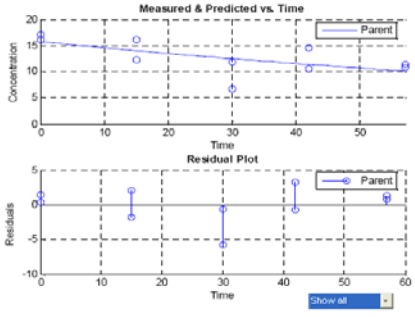
The degradation curves obtained with SFO and FOMC models are very similar, the same can be said with regard to the residuals. Also the statistical evaluation of both fits returned the similar results, although the parameters were slightly better in case of SFO model. Additionally it was stated that the model parameters for FOMC fit - α and β were statistically not reliable – the CI values for them passed through zero. For this reason the SFO model should be considered as that returning the reliable persistence endpoints. These endpoints cannot be however used in modelling, as they represent dissipation of sulfoxaflor from water phase and not its degradation there. It was noted that the rounding procedure was used. Therefore it was decided to recalculate the DT_{50} and DT_{90} values using the degradation rate constant *k* reported in the table 5.11.2.5-11 (DAR Table B.8.4.3.2-11). The resulting kinetic endpoints, reported with two digits after the decimal point, are following: DT_{50} = 64.18 days, DT_{90} = 213.20 days.

Kinetic evaluation of the sediment phase data:

Two models were used in the kinetic examination of the data for sulfoxaflor obtained in the sediment phase: SFO and FOMC. The "top-down" approach was applied, with the "adjusted Time 0". This point was defined to be that where concentration of sulfoxaflor reached it maximum. The identified "adjusted time 0" point was DAT 46, where the concentration of sulfoxaflor in sediment reached its maximum - ~17%AR. Therefore DAT 46 was set to DAT 0 and the subsequent time points were adjusted appropriately.

The results of this examination are presented below, in graphical form on figure 5.11.2.5-6 (DAR Figure B.8.4.3.2-6) and in numerical form in the table 5.11.2.5-12 (DAR Table B.8.4.3.2-12).

SFO kinetic model



FOMC kinetic model

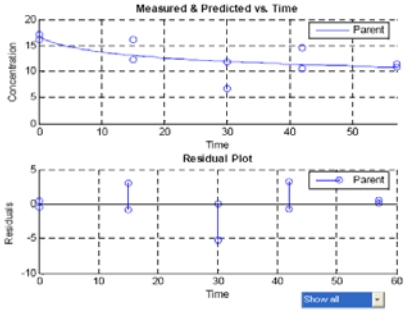


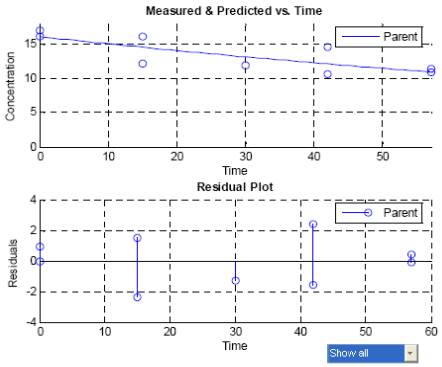
Figure 5.1.2.5-6 (DAR Figure B.8.4.3.2-6): The graphical results of the kinetic examination of the sediment phase data for sulfoxaflor obtained in the sand sediment system (M765).

Table 5.1.2.5-12 (DAR Table B.8.4.3.2-12): The numerical results of the kinetic examination of the sediment phase data for sulfoxaflor obtained in the sand sediment system (M765).

Kinetic model	Model parameters	Statistical evaluation of the parameter					Statistical evaluation of the fit		Kinetic endpoints (best-fit values)	
		Value	*Error*	*Confidence intervals*		*Prob. > t*	χ^2 *% error*	R^2	DT_{50} *[days]*	DT_{90} *[days]*
				Lower	Upper					
SFO	M_0	15.7	1.6	12.1	19.4	n. d.	10.3	0.3873	90	299
	k	0.0077	0.0033	-1.4 E-5	0.015	0.02052				
FOMC	M_0	16.7	1.9	12.2	21.1	n. d.	9.9	0.4686	300	>1000
	α	0.16	0.28	-0.49	0.82	0.2848				
	β	4	19	-40	49	0.4102				

Originally it was determined on examination that the results and stated that none of the models returned fully satisfying fit. Analysing the database more closely it was concluded that one of the replicates at DAT 76 (adjusted to DAT 30) – Replicate 2 (concentration of sulfoxaflor 6.7% AR), appeared to be an outlier and for this reason was removed from the data set. Then the kinetic examination was repeated. The results are shown below, in graphical form on figure 5.11.2.5-7 (DAR Figure B.8.4.3.2-7) and in numerical form in the table 5.11.2.5-13 (DAR Table B.8.4.3.2-13).

SFO kinetic model



FOMC kinetic model

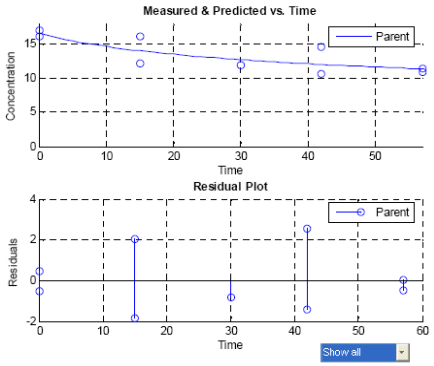


Figure 5.1.2.5-7 (DAR Figure B.8.4.3.2-7): The graphical results of the repeated kinetic examination of the sediment phase data for sulfoxaflor obtained in the sand sediment system (M765).

Table 5.1.2.5-13 (DAR Table B.8.4.3.2-13): The numerical results of the repeated kinetic examination of the sediment phase data for sulfoxaflor obtained in the sand sediment system (M765).

Kinetic model	Model parameters	Statistical evaluation of the parameter					Statistical evaluation of the fit		Kinetic endpoints (best-fit values)	
		Value	*Error*	*Confidence intervals*		*Prob. > t*	χ^2 *% error*	R^2	DT_{50} *[days]*	DT_{90} *[days]*
				Lower	Upper					
SFO	M_0	16.2	1.0	13.8	18.5	n. d.	4.0	0.6286	102	339
	k	0.0068	0.0020	0.0021	0.0115	0.0055				
FOMC	M_0	16.6	1.2	13.7	19.6	n. d.	3.2	0.6562	252	>1000
	α	0.24	0.36	-0.65	1.13	0.2672				
	β	15	42	-88	117	0.3683				

The degradation curves obtained with SFO and FOMC models are very similar, the same can be said with regard to the residuals. Also the statistical evaluation of both fits returned the similar results, although the parameters were slightly better in case of FOMC model. However, it was stated that the model parameters for FOMC fit - α and β were statistically not reliable – the CI values for them passed through zero. For this reason the SFO model should be considered as that

returning the reliable persistence and modelling endpoints. It was noted that the rounding procedure was used. Therefore it was decided to recalculate the DT_{50} and DT_{90} values using the degradation rate constant *k* reported in the table 5.11.2.5-13 (DAR Table B.8.4.3.2-13). The resulting kinetic endpoints, reported with two digits after the decimal point, are following: DT_{50} = 101.93 days, DT_{90} = 338.62 days.

Proposed endpoints:

The proposed kinetic endpoints are presented below in the table 5.1.2.5-14 (DAR Table B.8.4.3.2-14).

Table 5.1.2.5-14 (DAR Table B.8.4.3.2-14): The kinetic endpoints (persistence and modelling) proposed for sulfoxaflor in sand sediment system (M765).

Type of endpoints	Compartment:								
	Whole system			*Water phase*			*Sediment phase*		
	DT_{50} [days]	DT_{90} [days]	Kinetic model	DT_{50} [days]	DT_{90} [days]	Kinetic model	DT_{50} [days]	DT_{90} [days]	Kinetic model
Persistence	88.86	295.20	SFO	64.18	213.20	SFO	101.93	338.62	SFO
Modelling	88.86	295.20	SFO	n. d.	n. d.	----	101.93	338.62	SFO

b) The results of the kinetic evaluation of the data obtained in silt loam sediment system (M766):

Kinetic evaluation of the whole system (water/sediment) data:

Two models were used in the kinetic examination of the data for sulfoxaflor obtained in the whole system (water/sediment): SFO and FOMC. The results of this examination are presented below, in graphical form on figure 5.11.2.5-8 (DAR Figure B.8.4.3.2-8) and in numerical form in the table 5.11.2.5-15 (DAR Table B.8.4.3.2-15).

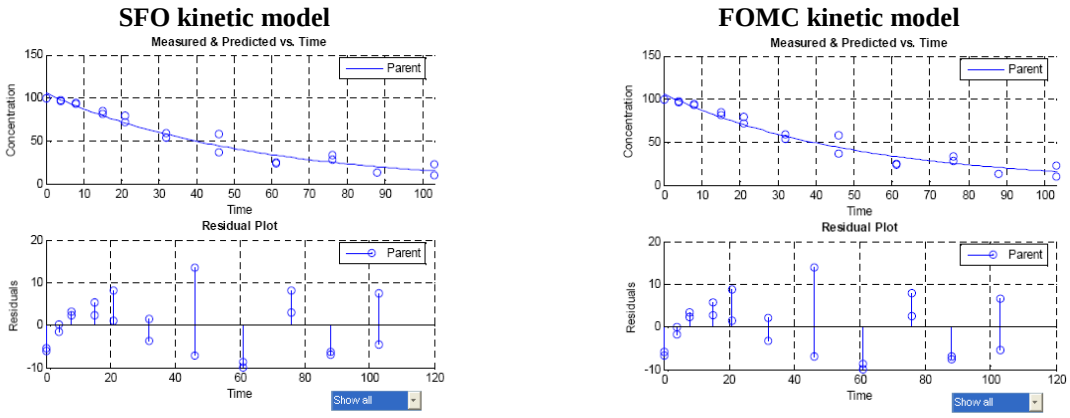


Figure 5.1.2.5-8 (DAR Figure B.8.4.3.2-8): The graphical results of the kinetic examination of the whole system data for sulfoxaflor obtained in the silt loam sediment system (M766).

Table 5.1.2.5-15 (DAR Table B.8.4.3.2-15): The numerical results of the kinetic examination of the whole system data for sulfoxaflor obtained in the silt loam sediment system (M766).

Kinetic model	Model parameters	Statistical evaluation of the parameter					Statistical evaluation of the fit		Kinetic endpoints (best-fit values)	
		Value	*Error*	*Confidence intervals*		*Prob. > t*	χ^2 *% error*	R^2	DT_{50} *[days]*	DT_{90} *[days]*
				Lower	Upper					
SFO	M_0	105.3	2.8	99.5	111.2	n. d	6.6	0.9629	37	122
	k	0.0189	0.0012	0.0165	0.0213	2.7 E-13				
FOMC	M_0	105.9	3.6	98.4	113.4	n. d.	7.2	0.9612	36	127
	α	13	40	-71	97	0.3753				
	β	656	> 1000	<-1000	> 1000	0.3810				

The degradation curves obtained with SFO and FOMC models are very similar, the same can be said with regard to the residuals. Also the statistical evaluation of both fits returned the similar results, although the parameters were better in case of SFO model. Additionally it was stated that the model parameters for FOMC fit - α and β were statistically not reliable – the CI values for them passed through zero. For this reason the SFO model should be considered as that returning the reliable persistence and modelling endpoints. It was noted that the rounding procedure was used. Therefore it was decided to recalculate the DT_{50} and DT_{90} values using the degradation rate constant *k* reported in the table 5.11.2.5-15 (DAR Table B.8.4.3.2-15). The resulting kinetic endpoints, reported with two digits after the decimal point, are following: DT_{50} = 36.67 days, DT_{90} = 121.83 days.

Kinetic evaluation of the water phase data:

Two models were initially used in the kinetic examination of the data for sulfoxaflor obtained in the water phase: SFO and FOMC. Subsequently, as FOMC returned better results, both in term of the visual fit and statistical evaluation, than the SFO, second bi-phasic model – DFOP, was tested. The results of this examination are presented below, in graphical form on figure 5.11.2.5-9 (DAR Figure B.8.4.3.2-9) and in numerical form in the table 5.11.2.5-16 (DAR Table B.8.4.3.2-16). It was declared that he adjusted the concentration at DAT 0 by adding the amount of sulfoxaflor found at that date in sediment to that reported for the water phase (this was done for both replicates).

SFO kinetic model **FOMC kinetic model** **DFOP kinetic model**

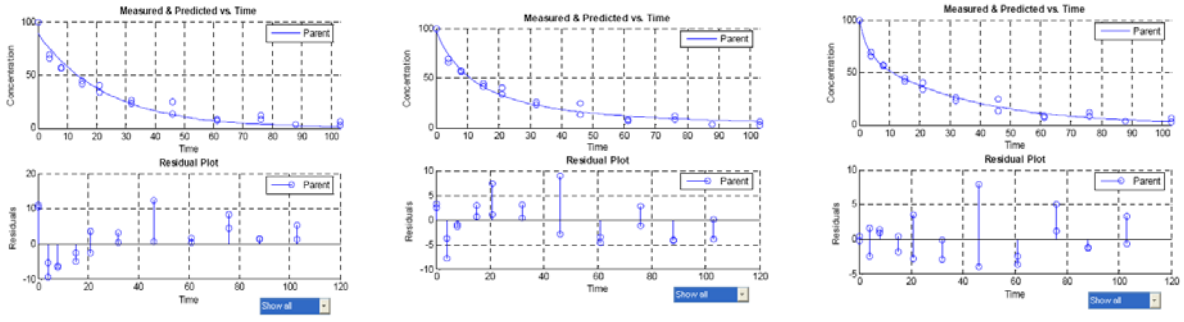


Figure 5.1.2.5-9 (DAR Figure B.8.4.3.2-9): The graphical results of the kinetic examination of the water phase data for sulfoxaflor obtained in the silt loam sediment system (M766).

Table 5.1.2.5-16 (DAR Table B.8.4.3.2-16): The numerical results of the kinetic examination of the water phase data for sulfoxaflor obtained in the silt loam sediment system (M766).

Kinetic model	Model parameters	Statistical evaluation of the parameter					Statistical evaluation of the fit		Kinetic endpoints (best-fit values)	
		Value	*Error*	*Confidence intervals*		*Prob. > t*	χ^2 *% error*	R^2	DT_{50} *[days]*	DT_{90} *[days]*
				Lower	Upper					
SFO	M_0	88.8	3.3	81.8	95.8	n. d.	13.0	0.9631	16	54
	k	0.0428	0.0035	0.0355	0.0501	4.6 E-11				
FOMC	M_0	96.7	2.9	90.7	102.7	n. d.	8.0	0.9817	11	72
	α	1.48	0.30	0.8610	2.1009	4.0 E-5				
	β	19.3	6.0	6.6	31.9	0.0024				
DFOP	M_0	99.68	2.3	94.8	104.3	n. d.	4.4	0.9903	11	63
	k_1	0.38	0.14	0.09	0.66	0.0062				
	k_2	0.031	0.002	0.025	0.036	1.4 E-10				
	g	0.31	0.05	0.21	0.41	1.9 E-6				

The visual inspection of the fits and the examination of the statistical data demonstrated that FOMC model returned better fit, both in statistical and visual terms. Therefore it was decided to test the second bi-phasic model – DFOP. This model gave better fit, visually and statistically, than the FOMC. Nevertheless the FOMC model was selected argumenting his choice by the fact, that when two models return equivalent results, it is appropriate to select the simpler one, in this case FOMC.

On analysing the results it was stated that R^2 was higher for DFOP, what indicated better fitting of the decline curve to the experimental data. Additionally the χ^2 error calculated for the DFOP fit was about two times lower (4.4) that that for the FOMC fit (8.0). Finally the residuals were slightly lower for the DFOP fit and more randomly distributed than it was in case of the FOMC fit.

All this suggests that rather DFOP should be selected as the best fit, despite its more complicated mathematical description.

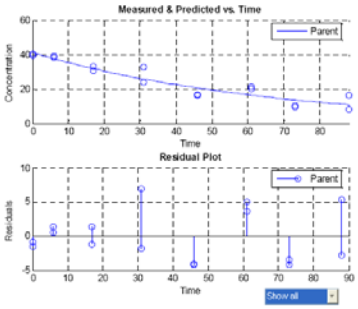
Additionally the proposal was to use the kinetic endpoints obtained with the SFO model as modelling endpoints. On review there was a disagreement with this proposal, mainly because the decline curves represent dissipation of sulfoxaflor from water phase (partly it is degradation, but migration to the sediment plays important role, more predominant than it was in case of sand sediment system) and not its degradation in water column. As a result, as it was in case of sand sediment system, for the water phase only persistence endpoints were derived. It was noted that the original submission used the rounding procedure, but decided not to recalculate them. Therefore the resulting kinetic endpoints are following: DT_{50} = 11 days, DT_{90} = 63 days.

Kinetic evaluation of the sediment phase data:

Two models were used in the kinetic examination of the data for sulfoxaflor obtained in the sediment phase: SFO and FOMC. The “top-down” approach was applied, with the “adjusted Time 0”. This point was defined to be that where concentration of sulfoxaflor reached its maximum. The identified “adjusted time 0” point was DAT 15, where the concentration of sulfoxaflor in sediment reached its maximum - ~40%AR. Therefore DAT 15 was set to DAT 0 and the subsequent time points were adjusted appropriately.

The results of this examination are presented below, in graphical form on figure 5.1.2.5-10 (DAR Figure B.8.4.3.2-10) and in numerical form in the table 5.1.2.5-17 (DAR Table B.8.4.3.2-17).

SFO kinetic model



FOMC kinetic model

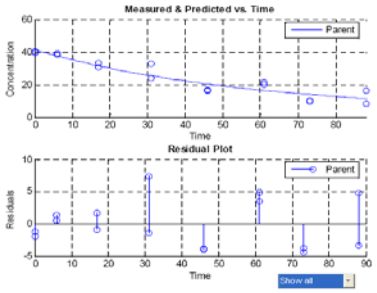


Figure 5.1.2.5.-10 (DAR Figure B.8.4.3.2-10): The graphical results of the kinetic examination of the sediment phase data for sulfoxaflor obtained in the silt loam sediment system (M766).

Table 5.1.2.5-17 (DAR Table B.8.4.3.2-17): The numerical results of the kinetic examination of the sediment phase data for sulfoxaflor obtained in the silt loam sediment system (M766).

Kinetic model	Model parameters	Statistical evaluation of the parameter					Statistical evaluation of the fit		Kinetic endpoints (best-fit values)	
		Value	*Error*	*Confidence intervals*		*Prob. > t*	*χ^2 % error*	*R^2*	*DT_{50} [days]*	*DT_{90} [days]*
				Lower	Upper					
SFO	M_0	41.4	1.9	37.4	45.5	n. d.	8.8	0.9006	46	153
	k	0.015	0.002	0.012	0.018	7.7 E-8				
FOMC	M_0	41.7	2.3	36.7	46.8	n. d.	9.6	0.8985	45	175
	α	5.2	16.9	-31	42	0.3812				
	β	315	> 1000	<-1000	> 1000	0.3916				

The degradation curves obtained with SFO and FOMC models are very similar, the same can be said with regard to the residuals. Also the statistical evaluation of both fits returned the similar results, although the parameters were better in case of SFO model. Additionally it was stated that the model parameters for FOMC fit - α and β were statistically not reliable – the CI values for them passed through zero. For this reason the SFO model should be considered as that returning the reliable persistence and modelling endpoints. It was noted that the the rounding procedure was used. Therefore it was decided to recalculate the DT_{50} and DT_{90} values using the degradation rate constant *k* reported in the table 5.11.2.5-17 (DAR Table B.8.4.3.2-17). The resulting kinetic endpoints, reported with two digits after the decimal point, are following: DT_{50} = 46.21 days, DT_{90} = 153.51 days.

Proposed endpoints:

The proposed kinetic endpoints are presented below in the table 5.1.2.5-18 (DAR Table B.8.4.3.2-18).

Table 5.1.2.5-18 (DAR Table B.8.4.3.2-18): The kinetic endpoints (persistence and modelling) proposed for sulfoxaflor in silt loam sediment system (M766).

Type of endpoints	Compartment:								
	Whole system			*Water phase*			*Sediment phase*		
	DT_{50} [days]	DT_{90} [days]	Kinetic model	DT_{50} [days]	DT_{90} [days]	Kinetic model	DT_{50} [days]	DT_{90} [days]	Kinetic model
Persistence	36.67	121.83	SFO	11	63	DFOP	46.21	153.51	SFO
Modelling	36.67	121.83	SFO	n. d.	n. d.	----	46.21	153.51	SFO

Conclusions:

The key endpoints from the water/sediment study are presented below in the tables 5.1.2.5-19 – 5.1.2.5-22 (DAR Tables B.8.4.3.2-19 – B.8.4.3.2-21).

Table 5.1.2.5-19 (DAR Table B.8.4.3.2-19): Distribution of the Applied Radioactivity (AR) in the system.

Water/ Sediment system	Characteristic of the system:			AR distribution in the system [%]:				Identified metabolites
				Max. in water phase	*Max. in sediment - extractable*	*NER*	*Mineralisation level ($^{14}CO_2$)*	
Sand sediment system – M765	***Sediment's texture class - USDA***		sand	98.1 (95.6 – 100.6) DAT 0	20.40 (19.5 – 21.3) DAT 103	6.55 (6.4 – 6.7) DAT 103	0.55 (0.5 – 0.6) DAT 103	X11719474
	pH	Water phase	6.7					
		Sediment	6.3					
	OC content	Water phase [ppm]	6.2					
		Sediment [%]	0.6					
	Incubation temperature [^{0}C]		20					
Silt loam sediment system – M766	***Sediment's texture class - USDA***		silt loam	99.3 (98.7 – 99.9) DAT 0	46.05 (45.4 – 46.7) DAT 32	24.35 (23.0 – 25.7) DAT 103	1.6 (1.5 – 1.7) DAT 88	X11719474
	pH	Water phase	7.8					
		Sediment	7.8					
	OC content	Water phase [ppm]	6.5					
		Sediment [%]	3.9					
	Incubation temperature [^{0}C]		20					

Table 5.1.2.5-20 (DAR Table B.8.4.3.2-20): Distribution of the sulfoxaflor and X11719474 in the system (% AR).

Water/ Sediment system	Characteristic of the system:			Distribution of sulfoxaflor in the system		Distribution of X11719474 in the system		
				Max. in water phase [%AR]	*Max. in sediment [% AR]*	*Max. in the system [%AR]*	*Max. in water phase [%AR]*	*Max. in sediment [% AR]*
Sand sediment system – M765	***Sediment's texture class - USDA***		sand	97.8 (95.6 – 100.0) DAT 0	16.6 (16.1 – 17.1) DAT 46	57.95 (45.0 – 70.9) DAT 76	48.05 (37.9 – 58.2) DAT 76	9.9 (7.1 – 12.7) DAT 76
	pH	Water phase	6.7					
		Sediment	6.3					
	OC content	Water phase [ppm]	6.2					
		Sediment [%]	0.6					
	Incubation temperature [^{0}C]		20					
Silt loam sediment system – M766	***Sediment's texture class - USDA***		silt loam	98.75 (97.9 – 99.6) DAT 0	40.15 (39.8 – 40.5) DAT 15	65.55 (65.1 – 66.0) DAT 88	35.25 (34.6 – 35.9) DAT 88	30.3 (30.1 – 30.5) DAT 88
	pH	Water phase	7.8					
		Sediment	7.8					
	OC content	Water phase [ppm]	6.5					
		Sediment [%]	3.9					
	Incubation temperature [^{0}C]		20					

Table 5.1.2.5-21 (DAR Table B.8.4.3.2-21): Kinetic endpoints determined for sulfoxaflor in water/sediment study.

Persistence endpoints:													
Water/ Sediment system	**Characteristic of the system:**			**Kinetic endpoints**								
				Whole system			***Water phase***			***Sediment***		
				DT_{50} [days]	DT_{90} [days]	Kinetic model	DT_{50} [days]	DT_{90} [days]	Kinetic model	DT_{50} [days]	DT_{90} [days]	Kinetic model
Sand sediment system – M765	***Sediment's texture class - USDA***		sand	88.86	295.20	SFO; χ^2 err = 7.0; R^2 = 0.8295	61.18	213.20	SFO; χ^2 err = 6.7; R^2 = 0.9013	101.93	388.62	SFO; χ^2 err = 4.0; R^2 = 0.6562
	pH	Water phase	6.7									
		Sediment	6.3									
	OC content	Water phase [ppm]	6.2									
		Sediment [%]	0.6									
	Incubation temperature [^{0}C]		20									
Silt loam sediment system – M766	***Sediment's texture class - USDA***		silt loam	36.67	121.83	SFO; χ^2 err = 6.6; R^2 = 0.9629	11	63	DFOP; χ^2 err = 4.4; R^2 = 0.9903	46.21	153.51	SFO; χ^2 err = 8.8; R^2 = 0.9006
	pH	Water phase	7.8									
		Sediment	7.8									
	OC content	Water phase [ppm]	6.5									
		Sediment [%]	3.9									
	Incubation temperature [^{0}C]		20									
Modelling endpoints:													
Water/ Sediment system	**Characteristic of the system:**			**Kinetic endpoints**								
				Whole system			***Water phase***			***Sediment***		
				DT_{50} [days]	DT_{90} [days]	Kinetic model	DT_{50} [days]	DT_{90} [days]	Kinetic model	DT_{50} [days]	DT_{90} [days]	Kinetic model
Sand sediment system – M765	***Sediment's texture class - USDA***		sand	88.86	295.20	SFO; χ^2 err = 7.0; R^2 = 0.8295	Not determined - dissipation	Not determined - dissipation	Not determined - dissipation	101.93	388.62	SFO; χ^2 err = 4.0; R^2 = 0.6562
	pH	Water phase	6.7									
		Sediment	6.3									
	OC content	Water phase [ppm]	6.2									
		Sediment [%]	0.6									
	Incubation temperature [^{0}C]		20									
Silt loam sediment system – M766	***Sediment's texture class - USDA***		silt loam	36.67	121.83	SFO; χ^2 err = 6.6; R^2 = 0.9629	Not determined - dissipation	Not determined - dissipation	Not determined - dissipation	46.21	153.51	SFO; χ^2 err = 8.8; R^2 = 0.9006
	pH	Water phase	7.8									
		Sediment	7.8									
	OC content	Water phase [ppm]	6.5									
		Sediment [%]	3.9									
	Incubation temperature [^{0}C]		20									
Geomean value				**57.08**	**189.63**		**----**	**----**		**68.63**	**244.25**	

The kinetic endpoints for the metabolite X11719474 were not determined as the distinct decline phase was not reached for this compound by the end of the study.

5.1.3 Summary and discussion of degradation

In the aquatic environment Sulfoxaflor was demonstrated to be hydrolytically and photolytically stable in the whole range of environmentally relevant pH (5-9) – for the aqueous hydrolysis DT_{50} >1000 days, for the direct aqueous photolysis DT_{50} = 489 days and for the indirect aqueous photolysis DT_{50} = 224 days. It can be therefore concluded that in none of the abiotic processes at least 70% of sulfoxaflor degraded within 28 days, so this compound cannot be considered rapidly degradable in abiotic processes in water. In the study on ready biodegradability it was demonstrated that within 28 days only up to 2.5% of it underwent mineralization, while at the same time the reference compound was mineralized completely. Therefore sulfoxaflor does not meet biodegradability criterion, i.e at least 70% mineralization within 28 days. This observation was confirmed by the results of the study on the degradation in biologically viable aquatic system (water/sediment study), in which only up to 1.6% of it was mineralized by the end of the study (on day 88th). On this basis it can be stated that sulfoxaflor **is not ready biodegradable** Finally, in the same study on the degradation in biologically viable aquatic system (water/sediment study) it was demonstrated that the average (geomean) DT_{50} for this compound was 57.08 days (the whole system value), therefore within 28 days much less than 70% of it undergoes biological degradation. As a result it can be stated that sulfoxaflor **is not rapidly biologically degradable.**

The final conclusion on the degradation of sulfoxaflor in the environment is that this compound is neither readily biodegradable nor rapidly degradable in the environment.

5.2 Environmental distribution

Environmental distribution of Sulfoxaflor was determined in batch sorption study, for the soil compartment, and in water/sediment study. For the air compartment the distribution was estimated on the basis of saturated vapour pressure values and Henry's law constants.

5.2.1 Adsorption/Desorption

The adsorption and desorption of Sulfoxaflor in soil was extensively examined in 17 soils (**Yoder R. N., Liu D., 2010; study report No. 080161**). The following observations were made as a result of this examination:
Sulfoxaflor is weakly sorbed onto soil with the average K_f = 0.47 mL/g (0.16 – 1.28 mL/g), average K_{fOC} = 35 mL/g (12 – 71 mL/g) and average 1/n = 0.96 (0.89 – 1.06). Such 1/n values indicate lack of any preferential mechanism of sorption, other than the affinity to the soil organic matter. It was also stated the adsorption of Sulfoxaflor onto soil is not pH-dependent.
The adsorption of Sulfoxaflor is only partly reversible; this was demonstrated in the desorption experiment. The desorption parameters are following: average K_f^{des} = 3.03 mL/g (1.20 – 7.24 mL/g), average K_{fOC}^{des} = 247 mL/g (55 – 613 mL/g) and average $1/n^{des}$ = 0.98 (0.83 – 1.13), what indicates no strong specific binding of this compound to any soil constituents.
The detailed information on the adsorption parameters for Sulfoxaflor is presented in the table 5.12.1-1 below.

Table 5.2.1-1: The soil adsorption constants for sulfoxaflor

Soil				**Adsorption parameters**					
Soil name	***Soil type (USDA classification)***	***pH***	***OC [%]***	**Distribution constants**		***Freundlich isotherm's parameters***			
				K_d [mL/g]	K_{dOC} [mL/g]	K_f [mL/g]	K_{fOC} [mL/g]	1/n	R^2
M761 – Cranwell	Loamy sand	7.6	1.3	***0.29***	***22.31***	0.29	22	1.06	0.966
M762 – Aberford	Loam	7.3	6.7	***0.93***	***13.88***	0.81	12	0.96	0.999
M763 – Malham	Silt loam	6.2	3.5	***0.47***	***13.43***	0.40	12	0.95	0.999
M764 – LUFA 5M	Sandy loam	7.4	1.2	***0.32***	***26.67***	0.30	25	1.02	0.997
M768 – Lenawee	Clay loam	5.9	1.8	***0.66***	***36.67***	0.56	31	0.96	1.000
M770 – Pullman (2)	Clay loam	6.9	1.2	***0.61***	***50.83***	0.57	47	0.99	1.000
M771 – Fayette	Loam	6.3	1.1	***0.63***	***57.27***	0.54	49	0.96	1.000
M772 – Slagle	Sandy loam	6.4	1.0	***0.37***	***37.00***	0.33	33	0.98	0.998
M775 – Italy	Sandy clay loam	7.4	1.3	***0.45***	***34.62***	0.40	31	0.97	0.999
M776 – Spain	Clay loam	7.8	1.2	***0.37***	***30.83***	0.35	30	1.00	0.996
M780 – France	Clay loam	7.8	1.7	***0.43***	***25.29***	0.34	20	0.95	0.993
M781 – Germany	Silt loam	6.3	1.1	***0.31***	***28.18***	0.26	24	0.93	0.998
M773 – California	Sand	6.3	0.3	***0.25***	***83.33***	0.16	54	0.89	0.964
M774 – Florida	Loamy sand	6.2	0.8	***0.57***	***71.25***	0.43	53	0.91	0.999
M777 – Bearden-Lindaas	Clay	7.9	1.8	***1.29***	***71.67***	1.28	71	0.98	1.000
M778 – Pullman (3)	Clay loam	6.7	1.1	***0.58***	***52.73***	0.51	46	0.97	1.000
M779 – Lacustrine	Loam	6.9	1.8	***0.68***	***37.78***	0.52	29	0.93	0.998
AVERAGE				***0.54***	***40.81***	**0.47**	**35**	**0.96**	**0.995**
SD				***0.26***	***20.61***	**0.26**	**16**	**0.04**	**0.011**
Minimum				***0.25***	***13.43***	**0.16**	**12**	**0.89**	**0.964**
Maximum				***1.29***	***83.33***	**1.28**	**71**	**1.06**	**1.000**

As it was stated that the soil sorption in soil of Sulfoxaflor was extensively examined in batch sorption studies and gave sufficient information as to the mobility of this compound in soil, no other experiments on the mobility of Sulfoxaflor in soil, such as column leaching studies, aged residues column leaching studies, lysimeter studies or field leaching studies were performed. Such approach was considered acceptable.

The results of batch sorption study indicate that Sulfoxaflor in the aquatic environment would be present mainly in the water phase, where it would undergo further transformation. The results of water/sediment study (**Laughlin L. A, Adelfinskaya Y., Balcer J. L, 2010; study report No. 080138**) confirm this statement. They are presented in tables below.

Table 5.2.1-2: Distribution of the Applied Radioactivity (AR) in the system

Water/ Sediment system	Characteristic of the system:			AR distribution in the system [%]:				Identified metabolites
				Max. in water phase	*Max. in sediment - extractable*	*NER*	*Mineralisation level ($^{14}CO_2$)*	
Sand sediment system – M765	*Sediment's texture class - USDA*		sand	98.1 (95.6 – 100.6) DAT 0	20.40 (19.5 – 21.3) DAT 103	6.55 (6.4 – 6.7) DAT 103	0.55 (0.5 – 0.6) DAT 103	X11719474
	pH	Water phase	6.7					
		Sediment	6.3					
	OC content	Water phase [ppm]	6.2					
		Sediment [%]	0.6					
	Incubation temperature [^{0}C]		20					
Silt loam sediment system – M766	*Sediment's texture class - USDA*		silt loam	99.3 (98.7 – 99.9) DAT 0	46.05 (45.4 – 46.7) DAT 32	24.35 (23.0 – 25.7) DAT 103	1.6 (1.5 – 1.7) DAT 88	X11719474
	pH	Water phase	7.8					
		Sediment	7.8					
	OC content	Water phase [ppm]	6.5					
		Sediment [%]	3.9					
	Incubation temperature [^{0}C]		20					

Table 5.2.1-3: Distribution of the sulfoxaflor and X11719474 in the system (% AR)

Water/ Sediment system	Characteristic of the system:			Distribution of sulfoxaflor in the system		Distribution of X11719474 in the system		
				Max. in water phase [%AR]	*Max. in sediment [% AR]*	*Max. in the system [%AR]*	*Max. in water phase [%AR]*	*Max. in sediment [% AR]*
Sand sediment system – M765	*Sediment's texture class - USDA*		sand	97.8 (95.6 – 100.0) DAT 0	16.6 (16.1 – 17.1) DAT 46	57.95 (45.0 – 70.9) DAT 76	48.05 (37.9 – 58.2) DAT 76	9.9 (7.1 – 12.7) DAT 76
	pH	Water phase	6.7					
		Sediment	6.3					
	OC content	Water phase [ppm]	6.2					
		Sediment [%]	0.6					
	Incubation temperature [^{0}C]		20					
Silt loam sediment system – M766	*Sediment's texture class - USDA*		silt loam	98.75 (97.9 – 99.6) DAT 0	40.15 (39.8 – 40.5) DAT 15	65.55 (65.1 – 66.0) DAT 88	35.25 (34.6 – 35.9) DAT 88	30.3 (30.1 – 30.5) DAT 88

5.2.2 Volatilisation

The volatility of Sulfoxaflor, expressed as its vapour pressure at ambient temperature (T = 20^{0}C) and Henry's law constant, are reported in the table below, together with its water solubility different pH.

Table 5.2.2-1: The data on the volatility of sulfoxaflor.

Parameter	Measurement conditions	Results obtained for: Sulfoxaflor (XDE-208)
Saturated vapour pressure – V_P [Pa]	***T = 20^{0}C***	≤ 1.4 E-6
	T = 25^{0}C	≤ 2.5 E-6 (*extrapolated*)
Solubility in water – *s* [mg/L]	***Purified water, T = 20^{0}C***	673
	pH = 5; T = 20^{0}C	1308
	pH = 7; T = 20^{0}C	568
	pH = 9; T = 20^{0}C	551
Henry's law constant [Pa*m^3*mol^{-1}]	***Unbuffered***	5.77 E-7
	pH 5	2.81 E-7
	pH7	6.83 E-7
	pH9	7.05 E-7

Additionally the rate of photochemical oxidation in air was determined for Sulfoxaflor using the Atkinson's method (**Weldenburg B. M., Boulton J. P., 2010; study report No. 101449**). The calculations were done using the EPI ver. 4.00 modelling tool. Firstly the overall degradation rate constant for Sulfoxaflor was calculated, which was subsequently used to determine the half life for the photochemical oxidation for Sulfoxaflor. - $t_{½}$. This was done using the assumed concentration of hydroxyl radicals in the atmosphere of 1.5 E6 [radicals/cm^3]. The calculated $t_{½}$ value was in turn used to calculate the Sulfoxaflor's DT_{50} for the process of photochemical oxidation in atmosphere assuming 12 hours of sunlight per day (24 hours).

The obtained results were following:
- overall degradation rate constant *k* = 16.5365 E-12 [cm^3*molecule^{-1}*sec^{-1}]
- half life for the photochemical oxidation $t_{½}$ = 7.762 [hours];
- photochemical oxidation DT_{50} = 0.647 [days] (for 12 hours of sunlight per day).

On the basis of the results presented above it can be stated that:
- Sulfoxaflor is a non-volatile compound (according to the EPPO classification presented in the FOCUS Air Guidance Document ; Focus 2005), which is expected to be short-living in the atmosphere;

As a result this compound is not expected to pose any serious threat to the atmosphere. For this reason the volatilisation from soil and plant surfaces was not examined for it.

5.2.3 Distribution modelling

Not performed

5.3 Aquatic Bioaccumulation

Analysing the physical-chemical properties of Sulfoxaflor, as well as its sorptive behaviour the compound has a very low affinity to organic compounds in general and lipids in particular.

Its solubility in water (unbuffered pure water at C = 20°C) is high for an organic compound - 670.3 mg/L. The Log Pow (20°C (99.7%)) is at pH 5: Log Pow= 0.806; at pH 7: Log Pow= 0.802; and at pH 9: Log Pow= 0.799, indicating that Sulfoxaflor has low or even very low affinity to lipids and other non-polar organic compounds (hence low expected bioaccumulation potential).

The results of the water/sediment studies indicate that this compound should be expected to occur mainly in the water phase. This is confirmed, although indirectly, by the soil adsorption constants.

As a consequence, Sulfoxaflor exhibits low bioaccumulation potential in either aquatic plants or aquatic animals because of the low affinity to lipids and, probably lignins. The same concerns, X11719474 and other major metabolites.

Table 22: Summary of relevant information on aquatic bioaccumulation

Method	Results	Remarks	Reference
Not applicable	No experimental data are available.	Not applicable	Not applicable

5.3.1 Aquatic bioaccumulation

5.3.1.1 Bioaccumulation estimation

The log Pow of Sulfoxaflor was found to be 0.799 - 0.806 at 20°C. Hence no bioconcentration study is demanded.

5.3.1.2 Measured bioaccumulation data

No experimental data are available.

5.3.2 Summary and discussion of aquatic bioaccumulation

Based on the measured log POW (0.799 - 0.806 at 20 °C) XDE-208 is considered to have a low bioaccumulation potential.

5.4 Aquatic Toxicity

Table 23: Summary of relevant information on aquatic toxicity

Method	Test organism	Test design	Results (mg a.s./L)			Remarks	Reference
			Endpoints	NOEC [mg/L]	LC_{50}/EC_{50} [mg/L]		
OECD 203, EPA 72-1, OPPTS 850.1075	Rainbow trout (*Oncorhynchus mykiss*)	acute, 96h, static	mortality sublethal effects	387	>387	mm	Gerke, A. 2008a
OECD 203, EPA 72-1, OPPTS 850.1075	Bluegill sunfish (*Lepomis macrochirus*)	acute, 96h, static	mortality subleth. effects	181	>316	im	Gerke, A. 2008b
OECD 203, EPA 72-1, OPPTS 850.1075	Common carp (*Cyprinus carpio*)	acute, 96h, static	mortality subleth. effects	402	>402	mm	Gerke, A. 2008c
OECD 203, OPPTS 850.1075	Sheepshead minnow (*Cyprinodon variegatus*)	acute, 96h, static	mortality subleth. effects	96.3	266	mm	Gerke, A. 2008d
OECD 210, OPPTS 850.1400	Fathead minnow (*Pimephales promelas*)	chronic, 30d ELS flow-through	egg hatchability fry survival length weight	5.05	12.9	mm	Boettcher M, Wydra, V 2009
OPPTS 850.1400	Sheepshead minnow (*Cyprinodon variegatus*)	chronic, 38d ELS flow-through	egg hatchability fry survival length weight	1.21	-	mm	Hicks, S.L. 2010
OECD 202, OPPTS 850.1010	*Daphnia magna*	acute, 48h, static	immobility	110	>399	mm	Hicks S.L. 2008a
OPPTS 850.1035, EPA 72-1	Mysid shrimp (*Americamysis bahia*)	acute, 96h, static	mortality subleth. effects	0.389	0.643	mm	Hicks S.L. 2008b
OECD 211, OPPTS 850.1300	*Daphnia magna*	chronic, 21d, semi-static	mortality reproduction growth	50	-	nom	Kuhl, R, Wydra, V. 2009a
OPPTS 850.1350, 850.1000 EPA 72-3	Mysid shrimp (*Americamysis bahia*)	chronic, 28d flow-through	mortality length subleth. effects	0.114	-	mm	Lehman, Ch. 2010
OECD 201, OPPTS 850.5400	Freshwater green (*Pseudokirchneriella subcapitata*)	growth inhibition, 96h, static	biomass yield growth rate	100	>100 >100 >100	nom	Dengler, D. 2009a
OECD 201, OPPTS 850.5400	Saltwater diatom (*Skeletonema*	growth inhibition, 96h, static	biomass yield	109	>109 >109	mm	Dengler, D. 2009b

Method	Test organism	Test design	Results (mg a.s./L)			Remarks	Reference
			Endpoints	NOEC [mg/L]	LC_{50}/EC_{50} [mg/L]		
	costatum)		growth rate		>109		
OECD 201, OPPTS 850.5400	Freshwater cyanobacteria (*Anabaena flos-aquae*)	growth inhibition, 96h, static	biomass yield growth rate	13	>98.3 >91.2 >104	mm	Dengler, D. 2009c
OECD 201, OPPTS 850.5400	Freshwater diatom (*Navicula pelliculosa*)	growth inhibition, 96h, static	biomass yield growth rate	3.7	85.7 >101 >101	mm	Dengler, D. 2009d
OECD 221, OPPTS 850.4400	Duckweed (*Lemna gibba*)	growth inhibition, 7d, semi-static	biomass frond yield growth rate	100 100 100	>100 >100 >100	nom	Kuhl, R, Wydra, V. 2009b
OPPTS 850.1025, EPA 72-3	Eastern oyster (*Crassostrea virginica*)	acute, 96h flow-through	shell growth	67.3	86.5	mm	Hicks S.L. 2008c
OECD 202, OPPTS 850.1010	*Chironomus dilutus*	acute, 96h, spiked water, static	mortality subleth. effects	<0.131	0.622	mm	Gerke, A. 2008e
OPPTS 850.1735	*Chironomus dilutus*	acute, 10d, spiked sediment, static	mortality weight	0.036	0.119	mm	Gerke, A. 2008f
OECD 219	*Chironomus riparius*	chronic, 28d, spiked water, static	survival emergence	0.0384	-	im	Gerke, A. 2009

mm – mean measured concentration
im – initial measured concentration
nom – nominal concentration

5.4.1 Fish

Short-term toxicity to fish

Study 1: Toxicity to fish (Sulfoxaflor DAR, Volume 3 - B.9.2.1.1.i)

Method	Test organism	Test design	Results (mg a.s./L)			Remarks	Reference
			Endpoints	NOEC [mg/L]	LC_{50}/EC_{50} [mg/L]		
OECD 203,	Rainbow trout	acute,	mortality subleth. effects	387	>387	mm	Gerke, A. 2008a

Method	Test organism	Test design	Results (mg a.s./L)			Remarks	Reference
			Endpoints	NOEC [mg/L]	LC_{50}/EC_{50} [mg/L]		
EPA 72-1, OPPTS 850.1075	(*Oncorhynchus mykiss*)	96h, static					

Acute toxicity to cold water fish: rainbow trout (*Oncorhynchus mykiss*)
Citation: Gerke, A. 2008a: Sulfoxaflor: Acute Toxicity Test to the Rainbow Trout, *Oncorhynchus mykiss*, Determined Under Static Test Conditions. ABC Laboratories, Columbia, Missouri, ABC Study Number 63661. Dow AgroSciences unpublished report, Study Number 080064. 27 August, 2008.

Guidelines: OECD guideline 203

OPPTS Number 850.1075

FIFRA Subdivision E, Section 72-1

TSCA 797.1400

GLP compliance: Yes.

Test material:

Test item: Sulfoxaflor

Purity: 95.6% w/w

Description: White solid

Lot No./Batch No.: TSN003725-0001, E2162-34

Material and methods:

A 96-hour static test was performed with test concentrations of 0 (control), 25, 50, 100, 200, and 400 mg Sulfoxaflor/L. All solution preparations were corrected for the purity of the test substance. Ten juvenile fishes were present in each test chamber with two replicates per test treatment, resulting in 20 fish per test treatment. Observations for mortality and sublethal responses were made at 24, 48, 72, and 96 hours. Temperature, pH, and dissolved oxygen concentration were measured in each test chamber on a daily basis. In addition, a continuous record of the temperature from the water bath was also maintained. Alkalinity, hardness, and conductivity were measured in a sample of the dilution water at test initiation.

Statistical analysis: Due to mortality rates <50%, estimates of LC50 values and their 95% confidence limits and the no-observed-effect concentration (NOEC) were not calculated.

Results
Water quality parameters (pH, temperature, dissolved oxygen and total hardness) remained within acceptable testing limits for rainbow trout throughout the test.

The control and test substance solutions ≤ 50 mg XDE -208/L were clear and colourless with no

visible precipitate, surface film, or undissolved test substance throughout the test. The 100 and 200 mg XDE-208/L test treatment solutions had a surface film partially covering the surface of the solution at initiation but were clear and colourless with no visible precipitate, surface film, or undissolved test substance at 24 hours and through the remainder of the test. The 400 mg XDE-208/L test treatment solution had a surface film throughout the test and undissolved test substance after 24 hours and for the remainder of the test. The 400 mg XDE-208/L test solution appears to be at or slightly above the functional solubility of XDE-208 in this dilution water, based on the surface film and undissolved test substance noted during the test. However, because the mean measured concentrations closely approximated the nominal concentration of 400 mg/L, it may be concluded that the biological results at this treatment level should be considered a valid measure of the effects of XDE-208.

Analytical confirmation of the test substance, XDE-208, within the test solutions, was performed at 0 and 96 hours, using an ultra high performance liquid chromatographic/mass spectrometry (UPLC/MS/MS) system. The measured concentrations in the test substance treatment sample collected at 0 hour were 25.2, 51.4, 105, 223, and 398 mg XDE-208/L or 100 to 112% of the nominal concentrations, indicating the treatments were appropriately dosed at test initiation. The measured concentrations in the test substance treatment samples collected at 96 hours were 28.1, 51.6, 110, 213, and 376 mg/L or 94 to 112% of the nominal concentrations. The mean measured concentrations in the test solutions were 26.7, 51.5, 108, 218, and 387 mg/L or 97 to 109% of the nominal concentrations. These results indicate that XDE-208 was stable for 96 hours in the test solution.

After 96 hours of exposure, mortality was 0% in the 0, 25, 50, 100, 200, and 400 mg XDE-208/L treatments. Three fish were observed on the bottom of the test chamber in the 400 mg XDE-208/L treatment after 24 hours of exposure, and one fish in the 400 mg XDE-208/L treatment was observed on the bottom after 72 hours. No other sublethal observations were noted throughout the exposure.

Reliability of the study
The reported study is GLP compliant and conducted to a standard study protocol. It is noted that the water temperature during the test (11.7-13.0˚C) was not maintained within the recommended range for the test species in OECD 203 (13-17˚C). However, this deviation is considered minor since results for control organisms were within the expected range. The test results are in compliance with the guideline's validity criteria. It is acceptable for regulatory use.

Since the measured concentrations remained between 80 and 120% of the nominal concentrations SANCO/3268/2001 recommends that endpoints should normally be expressed in terms of nominal concentrations. Because the slight differences between nominal and mean measured concentrations will not fundamentally change the toxicity value, the use of toxicity endpoints based on mean measured concentrations is therefore considered to be acceptable for risk assessment purposes.

Based on mean measured concentrations, the regulatory endpoint is a 96-hour LC_{50} > 387 mg XDE-208/L, the highest test substance concentration tested.

Study 2: Acute toxicity to warm water fish: Bluegill sunfish (*Lepomis macrochirus*) (Sulfoxaflor DAR, Volume 3 - B.9.2.1.1.ii)

Method	Test organism	Test	Results (mg a.s./L)	Remarks	Reference

		design	**Endpoints**	**NOEC [mg/L]**	**LC_{50}/EC_{50} [mg/L]**		
OECD 203, EPA 72-1, OPPTS 850.1075	Bluegill sunfish (*Lepomis macrochirus*)	acute, 96h, static	mortality subleth. effects	181	>316	im	Gerke, A. 2008b

Citation: Gerke, A. 2008b: Sulfoxaflor: Acute Toxicity Test to the Bluegill Sunfish, *Lepomis macrochirus*, Determined Under Static Test Conditions. ABC Laboratories, Columbia, Missouri, ABC Study Number 63662. Dow AgroSciences unpublished report, Study Number 080065. 10 September 2008.

Guidelines: OECD guideline 203

OPPTS Number 850.1075

FIFRA Subdivision E, Section 72-1

TSCA 797.1400

GLP compliance: Yes.

Test material:

Test item: Sulfoxaflor

Purity: 95.6% w/w

Description: White solid

Lot No./Batch No.: TSN003725-0001, E2162-34

Material and methods:

A 96-hour static test was performed with test concentrations of 0 (control), 25, 50, 100, 200, and 400 mg Sulfoxaflor/L. All solution preparations were corrected for the purity of the test substance. Ten juvenile fishes were present in each test chamber with two replicates per test treatment, resulting in 20 fish per test treatment. Observations for mortality and sublethal responses were made at 24, 48, 72, and 96 hours. Temperature, pH, and dissolved oxygen concentration were measured in each test chamber on a daily basis. In addition, a continuous record of the temperature from the water bath was also maintained. Alkalinity, hardness, and conductivity were measured in a sample of the dilution water at test initiation.

Statistical analyses: All statistical analyzes were performed with SAS software. Due to mortality rates <50%, estimates of LC50 values and their 95% confidence limits were not calculated. The no-observed effect concentration (NOEC) was determined using Fisher's Exact Test.

Results
Water quality parameters (pH, temperature, dissolved oxygen and total hardness) remained within acceptable testing limits for bluegill sunfish throughout the test.

Analytical confirmation of the test substance, XDE-208, within the test solutions, was performed at 0 and 96 hours, using an ultra high performance liquid chromatographic/mass spectrometry (UPLC/MS/MS) system. The measured concentrations in the test substance treatment sample

collected at 0 hour were 24.8, 50.2, 95.5, 181, and 316 mg XDE-208/L or 79 to 100% of the nominal concentrations, indicating the treatments were appropriately dosed at test initiation. The measured concentrations in the test substance treatment samples collected at 96 hours were 24.1, 49.8, 104, 200, and 410 mg/L or 96 to 104% of the nominal concentrations. The mean measured concentrations in the test solutions were 24.5, 50.0, 99.8, 191, and 363 mg/L or 91 to 100% of the nominal concentrations.

After 96 hours of exposure, mortality was 0, 0, 0, 0, 0, and 5% in the 0 (control), 25, 50, 100, 200, and 400 mg XDE-208/L treatments, respectively. Sublethal observations included discoloration: 20% of fishes were observed to be discolored in the 400 mg XDE-208/L treatment after 48 hours of exposure, and 19% of fishes in the 400 mg XDE-208/L after 72 and 96 hours. No other sublethal observations were noted throughout the exposure.

Reliability of the study
The reported study is GLP compliant and conducted to a standard study protocol without significant deviations. The test results are in compliance with the guideline's validity criteria. It is acceptable for regulatory use.

Since the initial measured concentrations fall below 80% of nominal, the toxicity values should be expressed as initial measured concentrations (according to SANCO/3268/2001).

Based on initial measured concentration, the regulatory endpoint is 96-hour LC50 > 316 mg XDE 208/L, the highest concentration tested.

Study 3: Acute toxicity to warm water fish: Carp (*Cyprinus carpio*) (Sulfoxaflor DAR, Volume 3 - B.9.2.1.1.iii)

Method	Test organism	Test design	Results (mg a.s./L)			Remarks	Reference
			Endpoints	NOEC [mg/L]	LC_{50}/EC_{50} [mg/L]		
OECD 203, EPA 72-1, OPPTS 850.1075	Common carp (*Cyprinus carpio*)	acute, 96h, static	mortality subleth. effects	402	>402	mm	Gerke, A. 2008c

Citation: Gerke, A. 2008c: Sulfoxaflor: Acute Toxicity Test to the Common Carp, *Cyprinus carpio*, Determined Under Static Test Conditions. ABC Laboratories, Columbia, Missouri, ABC Study Number 63663. Dow AgroSciences unpublished report, Study Number 080066. 27 August 2008.

Guidelines: OECD guideline 203

OPPTS Number 850.1075

FIFRA Subdivision E, Section 72-1

TSCA 797.1400

JMAFF Guideline 2-7-1

GLP compliance: Yes.

Test material:

Test item: Sulfoxaflor

Purity: 95.6% w/w

Description: White solid

Lot No./Batch No.: TSN003725-0001, E2162-34

Material and methods:

A 96-hour static test was performed with test concentrations of 0 (control), 200, and 400 mg Sulfoxaflor/L. All solution preparations were corrected for the purity of the test substance. Ten juvenile fish were present in each test chamber with three replicates per test treatment, resulting in 30 fish per test treatment. Observations for mortality and sublethal responses were made at 24, 48, 72, and 96 hours. Note: The 200 mg Sulfoxaflor/L treatment level was dropped from the study due to disease (fin rot) observed in one replicate. Temperature, pH, and dissolved oxygen concentration were measured in each test chamber on a daily basis. In addition, a continuous record of the temperature from the water bath was also maintained. Alkalinity, hardness, and conductivity were measured in a sample of the dilution water at test initiation.

No statistical analysis was performed due to mortality rates <50%.

Results
Environmental parameters (pH, temperature, dissolved oxygen and total hardness) remained within acceptable limits throughout the duration of the study. All control and test substance solutions were clear and colourless with no visible precipitate, surface film, or undissolved test substance throughout the definitive test.

Analytical confirmation of the test substance, XDE-208, within the test solutions, was performed at 0 and 96 hours, using an ultra high performance liquid chromatographic/mass spectrometry (UPLC/MS/MS) system. The measured concentrations in the test substance treatment replicates collected at 0 hour were 405, 418, and 414 mg XDE-208/L or 101 to 105% of the nominal concentrations, indicating the treatments were appropriately dosed at test initiation. The measured concentrations in the test substance treatment samples collected at 96 hours were 386, 386, and 404 mg/L or 97 to 101% of the nominal concentrations. The mean measured concentration in the test solutions was 402 mg XDE-208/L or 101% of the nominal concentration. These results indicate that XDE-208 was stable for 96 hours in the test solution.

After 96 hours of exposure, mortality was 0 and 0% in the 0 (control) and 400 mg XDE-208/L treatments, respectively. A single replicate in the 200 mg XDE-208/L treatment had six fish die and the remaining four fish were noted to have fin rot. Due to this disease observed in a replicate, the 200 mg XDE-208/L treatment was considered invalid and is disregarded through this report. No other sublethal observations were noted.

Reliability of the study
The reported study is GLP compliant and conducted to a standard study protocol without significant deviations. The test results are in compliance with the guideline's validity criteria. It is acceptable for regulatory use.

Since the measured concentrations remained between 80 and 120% of the nominal concentrations SANCO/3268/2001 recommends that endpoints should normally be expressed in terms of nominal

concentrations. Because the slight differences between nominal and mean measured concentrations will not fundamentally change the toxicity value, the use of toxicity endpoints based on mean measured concentrations is therefore considered to be acceptable for risk assessment purposes.

Based on mean measured concentrations, the regulatory endpoint is a 96-hour LC_{50} > 402 mg XDE-208/L, the highest concentration tested.

Study 4: Acute toxicity to marine or estuarine fish: Sheepshead minnow (*Cyprinodon variegatus*) (Sulfoxaflor DAR, Volume 3 - B.9.2.1.1.iv)

Method	Test organism	Test design	Results (mg a.s./L)			Remarks	Reference
			Endpoints	NOEC [mg/L]	LC_{50}/EC_{50} [mg/L]		
OECD 203, OPPTS 850.1075	Sheepshead minnow (*Cyprinodon variegatus*)	acute, 96h, static	mortality subleth. effects	96.3	266	mm	Gerke, A. 2008d

Citation: Gerke, A. 2008g: Sulfoxaflor: Acute Toxicity Test to the Sheepshead Minnow, *Cyprinodon variegatus*, Determined Under Static Test Conditions. ABC Laboratories, Columbia, Missouri, ABC 63664. Dow AgroSciences unpublished report, Study Number 080067. August 27, 2008.

Guidelines: OECD guideline 203

OPPTS Number 850.1075

GLP compliance: Yes.

Test material:

Test item: Sulfoxaflor

Purity: 95.6% w/w

Description: White solid

Lot No./Batch No.: TSN003725-0001, E2162-34

Material and methods:

A 96-hour static test was performed with test concentrations of 0 (control), 25, 50, 100, 200, and 400 mg Sulfoxaflor/L. All solution preparations were corrected for the purity of the test substance. Ten juvenile fish were present in each test chamber with two replicates per test treatment, resulting in 20 fish per test treatment. Observations for mortality and sublethal responses were made at 24, 48, 72, and 96 hours.

Temperature, pH, dissolved oxygen concentration, and salinity were measured in each test chamber on a daily basis. In addition, a continuous record of the temperature from the water bath was also maintained.

Statistical analysis: All statistical analyses were performed with SAS software. Estimates of LC50 values and their 95% confidence limits were calculated using the probit method and Trimmed Spearman-Karber method. When the P value for Goodness of Fit was >0.05 and there was no other evidence of questionable convergence, the probit method was selected for reporting. When this

criterion was not achieved, the Trimmed Spearman-Karber method was selected for reporting. The no-observed effect concentration (NOEC) was determined by using a Fisher's exact test. A Hochberg adjustment was used to control the experiment wise error rate for the Fisher's test at the same alpha level.

Results
Water quality parameters remained within acceptable testing limits for sheepshead minnow throughout the test. All control and test substance solutions were clear and colourless with no visible precipitate, surface film, or undissolved test substance throughout the definitive test.

Analytical confirmation of the test substance, XDE-208, within the test solutions, was performed at 0 and 96 hours, using a high performance liquid chromatographic/mass spectrometry (HPLC/MS/MS) system. The measured concentrations in the test substance treatment sample collected at 0 hour were 23.6, 40.0, 94.5, 194, and 304 mg XDE-208/L or 76 to 97% of the nominal concentrations, indicating the treatments were appropriately dosed at test initiation. The measured concentrations in the test substance treatment samples collected at 96 hours were 23.5, 49.0, 98.0, 199, and 394 mg/L or 98 to 100% of the nominal concentrations. The mean measured concentrations in the test solutions were 23.6, 44.5, 96.3, 197, and 349 mg XDE-208/L or 87 to 99% of the nominal concentrations. These results indicate that XDE-208 was stable for 96 hours in the test solution. No residues of XDE-208 were in the control solutions above the MQL of 2.50 mg/L. Since the measured formulation concentrations approximated the nominal concentrations (i.e., within 80 to 120% of nominal) and were stable, the biological response results are based upon the nominal concentrations and the mean measured concentrations.

After 96 hours of exposure, mortality was 0, 0, 0, 0, 0, and 95% in the 0, 25, 50, 100, 200, and 400 mg XDE-208/L treatments (B.9.2.1.1). Sublethal observations included lying on the bottom of the test chamber and loss of equilibrium (B.9.2.1.2).

Table 5.4.1.1.Study 4.1 (DAR Table B.9.2.1.1) Effect of XDE-208 on mortality of sheepshead minnow

Treatment (mg a.i./L)		No. of Fish	Cumulative Mortality				
Nominal	Mean Measured		24-h	48-h	72-h	96-h	Total (%)
Negative control	<MQL	20	0	0	0	0	0
25	23.6	20	0	0	0	0	0
50	44.5	20	0	0	0	0	0
100	96.3	20	0	0	0	0	0
200	197	20	0	0	0	0	0
400	349	20	0	0	10	19	95
96 hour LC_{50}		288 mg a.i./L (nominal) or 266 mg a.i./L (mean measured)					
95% C.L.		277 to 299 mg a.i./L (nominal) or 258 to 275 mg a.i./L (mean measured)					
NOEC		100 mg a.i./L (nominal) or 96.3 mg a.i./L (mean measured)					

Table 5.4.1.1.Study 4.2 (DAR Table B.9.2.1.2) Sub-lethal effects of XDE-208 in sheepshead minnow

Treatment (mg a.i./L)	Observation Period

Nominal	Mean Measured	Loss of equilibrium (% affected)				Lying on bottom (% affected)			
		24-h	48-h	72-h	96-h	24-h	48-h	72-h	96-h
Negative control	<MQL	0	0	0	0	0	0	0	0
25	23.6	0	0	0	0	0	0	0	0
50	44.5	0	0	0	0	0	0	0	0
100	96.3	0	0	0	0	0	0	0	0
200	197	0	0	0	0	0	0	0	16 (80)
400	349	0	0	2 (20)	0	0	0	6 (60)	1 (100)

Reliability of the study
The reported study is GLP compliant and conducted to a standard study protocol without significant deviations. The test results are in compliance with the guideline's validity criteria. It is acceptable for regulatory use.

Since the initial measured concentrations fall below 80% of the nominal concentrations, the initial mean measured concentration is used in the risk assessment. However, the biological response results are based upon the nominal concentrations and the mean measured concentrations only. Since the mean measured concentrations remained close to the initial measured concentrations, it is acceptable to calculate toxicity endpoints using mean measured concentrations.

Based on mean measured concentrations, the regulatory endpoint is a 96-hour LC_{50} 266 mg XDE-208/L.

Long-term toxicity to fish

Study 1: Chronic toxicity to fish: Fathead Minnow (*Pimephales promelas*) Early life stage toxicity study (Sulfoxaflor DAR, Volume 3 - B.9.2.1.1.v)

Method	Test organism	Test design	Results (mg a.s./L)			Remarks	Reference
			Endpoints	NOEC [mg/L]	LC_{50}/EC_{50} [mg/L]		
OECD 210, OPPTS 850.1400	Fathead minnow (*Pimephales promelas*)	chronic, 30d ELS flow-through	egg hatchability fry survival length weight	5.05	12.9	mm	Boettcher M, Wydra, V 2009

Citation: Boettcher M, Wydra, V (2009): Sulfoxaflor Technical: Toxicity of Sulfoxaflor Technical to Fathead Minnow (Pimephales promelas) in an Early-Life Stage Test. Institut für Biologische Analytik und Consulting IBACON GmbH, Arheilger Weg 17, 64380 Rossdorf, Germany. IBACON Project Number: 46843232. Dow AgroSciences unpublished report, Study Number 080444. Study Report Completion July 13, 2009.

Guidelines: OECD guideline 210

OPPTS Number 850.1400

GLP compliance: Yes.

Test material:

Test item: Sulfoxaflor

Purity: 95.6% w/w

Description: White solid

Lot No./Batch No.: E2162-34

Material and methods:

A 30-day flow-through test was performed with test concentrations of 0 (control), 0.63, 1.25, 2.5, 5.0 and 10 mg Sulfoxaflor/L. All solution preparations were corrected for the purity of the test substance. Before initiating the biological part of the study, the test solutions were allowed to flow through the test aquaria for the equilibration period for 3 days. At the start of the test 30 fertilized eggs were present in each aquarium with two replicates per test treatment, resulting in 60 eggs per test treatment. When hatching commenced on day 1, the number of embryos hatched in each replicate was recorded daily until day 4. However, on day 4 more than 90% of the larvae were hatched in the controls. Therefore, day 4 was designated day 0 of the 30 day post-hatch period. During the test period the eggs and larvae were observed daily for survival, hatching, abnormal appearance and behaviour. Additionally, at the end of the test all surviving fish were weighted and their individual length was determined.

The stock solution (200 mg Sulfoxaflor/L) of the highest test concentration was prepared by dissolving the test item into the water by intense stirring for 4 hours. The intermediate stock solutions for each test concentration were prepared by dissolving adequate volumes of the stock solution of 200 mg Sulfoxaflor/L into the test water by intense stirring. The intermediate stock solutions and the test water were pumped in mixing vessels (one per replicate) with a constant flow rate by flexible-tube pumps, respectively. In these vessels the stock solutions and the test water were continuously mixed using a magnetic stirrer. Nominal test concentrations of 10, 5.0, 2.5, 1.25, 0.63 mg Sulfoxaflor/L did result. The mixing vessels and the aquaria were connected by a tube. The stock solutions were renewed after 3 - 4 days. Prior to the initiation of the test the dosing system was calibrated through the use of appropriate analysis techniques.

The flow rate of the stock solution and the dilution water were determined once a week during the test. From day two, the appearance of the test item in test water was determined daily in the test media of all test concentrations.

Duplicate samples from the test media (aquaria) of all test concentrations and the control and unicate samples from the stock solution were taken at day -1, 0 (=start of the test), 3, 7, 14, 21, 28 and 34. Additionally duplicate samples of the test concentration of 10 m/L were taken on day 4, as a dosage error occurred on day 3. All test medium samples were taken from the approximate centre of the aquaria.

Statistical Analysis: The hatching success was determined directly from the raw data. The NOEC and LOEC for mortality/survival were calculated by Fisher's Exact Test with Bonferroni Cossection, respectively. The NOEC calculation for body length and body weight was done by Dunnett's Multiplet- test Procedure. The NOEC was determined based on lack of statistically significant effects.

Deviations to the study plan:

1. In the test media of 10 mg test item/L a dosage error occurred by accident, which resulted in elevated concentrations of the test item in one aquaria and reduced concentrations in the second aquaria.
   Presumed effect on the study: Considering the biological results of the two aquaria this short-time incident did not influence the test.

2. The newly-hatched larvae should be fed with 24 h-old brine shrimp nauplii (Western Brine Shrimp International, Salt Lake City, USA). The juveniles should be fed with 48 h-old brine shrimp nauplii (Western Brine Shrimp International, Salt Lake City, USA). But the newly-hatched larvae were fed with JBL Nobil Fluid (JBL GmbH co. KG, 67141 Neuhofen, Germany) and 24 to 48-h old brine shrimp nauplii (Western Brine Shrimp International, Salt Lake City, USA) and the juveniles were fed with 24 to 48 h-old brine shrimp nauplii (Western Brine Shrimp International, Salt Lake City, USA).
   Presumed effect on the study: None. The controlled fish showed normal development.

3. The flow rate of the stock solution and the dilution water was determined once a week instead of weekly.
   Presumed effect on the study: None, since the analytical values clearly demonstrate that test item concentrations were in the nominal range during the test in all treatments except the highest treatment level.

4. The appearance of the test item in test water should be determined daily in the test media of all test concentrations, but it was not determined on day 0, 1 and 2.
   Presumed effect on the study: None, as the appearance of the test item was determined every day thereafter and showed no abnormalities.

5. At least one sample from the freshly prepared stock solutions and at least duplicate samples from the test media (aquaria) of all test concentrations and the control should be taken prior to the initiation of the test and afterwards at least once per week. But the stock solution was prepared on day -3 and only sampled on day -1.
   Presumed effect on the study: None, since the analytical values clearly demonstrate that test item concentrations were in the nominal range during the test.

Results
The validity criteria in terms of dissolved oxygen concentration, control mortality, hatching success and water temperature were satisfactorily maintained during the test.

The concentrations of the test item in solution were satisfactorily maintained within ± 20 % of the mean measured values apart from the test media of nominal 10 mg XDE-208/L. In this concentration level the samples collected at day 3 were outside the demanded range. In one aquaria of this treatment level the concentration was above the nominal concentration and below the nominal concentration in the second aquaria. Since the biological effects in both aquaria were comparable, this short-time incident was considered to be a minor deviation.

Hatching success
By Day 0 (post-hatch), more than 90% of the larvae were hatched in the controls. Hatching success was 100% at all test levels. Embryo development therefore appeared to be unaffected by the presence of XDE-208. Table B.9.2.1.3 shows the hatching success.

Table 5.4.1.2.Study 1.1 (DAR study B.9.2.1.3) Effects of XDE-208 on hatchability, survival and growth of fathead minnow.

Concentration (mg a.i./L) nominal/mean measured	No. of eggs at study initiation	% egg hatchability	No. of surviving fry	% fry survival	Mean length of surviving fish (cm)*	Mean wet weight of surviving fish (mg)*
Negative control	60	100	46	77	1.4	29.97
0.63 / 0.66	60	100	44	73	1.43	30.26
1.25 / 1.24	60	100	39	68	1.44	24.57
2.5 / 2.55	60	100	41	72	1.39	22.70
5.0 / 5.05	60	100	32	54	1.48	23.82
10 / 12.9	60	100	40	68	1.44	21.22
	Hatchability		Survival		Growth	
EC50 (mg a.i./L)	n.d.		> 10		n.d.	
NOEC (mg a.i./L)	≥ 10		≥ 10		5.0	
LOEC (mg a.i./L)	> 10		> 10		10	

Post-hatch survival
No significant mortality was observed in any test concentration or control. Table B.9.2.1.3 shows the percentage survival from day 0 until the end of the test.

Sub-lethal observations
No significant sublethal effects were observed at any treatment group.

Weight and length
The length of fish were unaffected by XDE-208. The weight was the most sensitive endpoint in this test. There were apparent effects at the 10 mg test item/L concentration. The mean weight and length parameters for surviving fry at the end of the test are shown in table B.9.2.1.3.

Reliability of the study
The reported study is GLP compliant and conducted to a standard study protocol. Several deviations to the study plan were noted, but they are considered minor and they are not supposed to result in any significant effects on the study. The test results are in compliance with the guideline's validity criteria. The study is acceptable for regulatory use.

Since the measured concentrations fall below 80% of the nominal concentrations, the mean measured concentrations are used in the risk assessment.

Based on mean measured concentrations, the regulatory endpoint is a 30-day NOEC is 5.05 mg XDE-208/L.

Study 2: Chronic toxicity to fish: Sheepshead minnow (*Cyprinodon variegatus*) Early life stage toxicity study (Sulfoxaflor DAR, Volume 3 - B.9.2.1.1.vi)

Method	Test organism	Test design	Results (mg a.s./L)			Remarks	Reference
			Endpoints	NOEC [mg/L]	LC_{50}/EC_{50} [mg/L]		
OPPTS 850.1400	Sheepshead minnow (*Cyprinodon variegatus*)	chronic, 38d ELS flow-through	egg hatchability fry survival length weight	1.21	-	mm	Hicks, S.L. 2010

Citation: Hicks, S.L. 2010: Sulfoxaflor: Early Life-Stage Toxicity Test with the Sheepshead Minnow, *Cyprinodon variegatus*, Under Flow-Through Conditions. ABC Laboratories, Columbia, Missouri, ABC 65667. Dow AgroSciences unpublished report, Study Number 101286. 14 June 2010.

Guidelines: OPPTS Number 850.1400

GLP compliance: Yes.

Test material:

Test item: Sulfoxaflor

Purity: 95.6% w/w

Description: White solid

Lot No./Batch No.: TSN003725-0001, E2162-34

Material and methods:

A 38-day flow-through test was performed with test concentrations of 0 (control), 0.65, 1.3, 2.5, 5.0 and 10 mg Sulfoxaflor/L. All solution preparations were corrected for the purity of the test substance. Diluter stock solutions were prepared at a target nominal concentration of 195 mg Sulfoxaflor/L at least once every eight days by diluting approximately 40.7950 g Sulfoxaflor/L (approximately 39.0000 g as active ingredient) to a volume of 200 L with dilution water in a stainless steel barrel. A 2-L proportional equal solvent diluter system, with an FMI metering pump, was used for the intermittent introduction of control and Sulfoxaflor test solutions into each test chamber during the test. The diluter cycle rate during the test was maintained at approximately 4.7 cycles/hour, which was sufficient to provide approximately 6.3 volume additions to each test chamber over a 24-hour period. At each cycle of the diluter system, the FMI pump introduced approximately 200-mL volumes of the diluter stock solution to the diluter system where the solution was diluted with approximately 3,700 mL of dilution water, resulting in a final solution volume of approximately 3,900 mL. Test chambers consisted of glass aquaria measuring approximately 18 cm wide by 20 cm long by 33 cm high with a test solution depth of 25 cm. These dimensions yielded a test solution volume of approximately 9 L. During the definitive testing, each treatment was replicated four times. Aquaria were arranged in a temperature-controlled water bath using a computer-generated random number table.

The test was initiated when a target number of 25 embryos were distributed to an egg cup (glass cups constructed from 9-cm diameter glass jars with Nitex® screen replacing the bottom and suspended within each replicate chamber) in each of four test chambers for the control and each test substance treatment, yielding a target number of 100 embryos per treatment group. To facilitate test

solution circulation, the cups were oscillated vertically in each chamber by means of a rocker arm apparatus driven by a low rpm electric motor. On a daily basis during incubation, the embryos were counted and dead embryos were removed and discarded. Day 0 post-hatch was based on ≥95% hatch in the control group (Study Day 8). On study day 14 (i.e., day 6 post-hatch), all live fry were counted and released into their respective replicate growth chamber. Embryos that had not yet hatched by the date of release were maintained in the egg cup until they had hatched, at which time they were released into their respective replicate growth chamber. Survival was monitored daily by visually inspecting each test chamber, and any behavioural or physical changes were recorded, including abnormalities. The test chambers were cleaned periodically (at least two times each week following the initial feeding) during the test to remove waste material and uneaten food and to minimize biological growth on the sides and bottom of the test chamber. After 30 days of post-hatch growth (Study Day 38), surviving fish were carefully netted from each replicate chamber and euthanized with tricaine methanesulfonate (MS-222; Western Chemical, Inc.). All individuals were measured for standard length (i.e., tip of the snout to the caudal peduncle) using a millimetre scale and blotted wet weight using an electronic balance.

Temperature, pH, salinity, and dissolved oxygen concentration were measured in all replicates of the test substance treatments and control groups at test initiation, weekly throughout the test, and at termination of the definitive test. No aeration was provided to any control or test substance chamber during the test. A continuous recording of temperature in a centrally located test chamber (control replicate C) was made using a datalogger and thermistor probe.

Statistical Analysis: All statistical analyses were performed using SAS software. Inferences of statistical significance were based upon a $p = 0.05$ unless otherwise noted. The no-observed-effect concentration (NOEC) and lowest-observed-effect concentration for egg hatchability and fish survival (30-Day post-hatch) data were determined by using a one-tailed Dunnett's test and a Fisher's exact test with the alternate hypothesis being the mean for the parameter was reduced in comparison to the pooled control mean. A Hochberg adjustment was used to control the experiment-wise error rate for the Fisher's test at the same alpha level. The NOEC and LOEC, based on standard length and blotted wet weight, were also estimated using a one-way analysis of variance (ANOVA) procedure and a one-tailed Dunnett's test with the alternate hypothesis being the mean for the parameter was reduced in comparison to the control mean. Prior to the Dunnett's test, a Shapiro-Wilk's test and a Levene's test were conducted to test for normality and homogeneity of variance, respectively, over treatments for each endpoint. Where possible, the point estimates of the maximum acceptable toxicant concentration (MATC) were calculated as the geometric mean of the NOEC and LOEC values of the sensitive endpoints.

Results

Water quality parameters remained within acceptable testing limits throughout the test. All test solutions were clear and colourless with no visible particulate material, surface film, undissolved test substance, or precipitate throughout the test.

Test solutions were analyzed for the concentration of XDE-208 using a liquid chromatography with tandem mass spectrometry (LC-MS/MS) system, the samples were collected prior to initiation (day –2) and on study days 0, 7, 14, 21, 28, 35, and 38 (termination) of the definitive test. Measured concentrations of XDE-208 in the test-substance treatments prior to initiation (i.e., day -2) of the definitive test were 0.545, 1.14, 2.42, 4.96, and 9.60 mg XDE-208/L and ranged from 84% to 99% of the nominal concentrations. Measured concentrations of XDE-208 in the test-substance treatments on day 0 of the exposure were 0.610, 1.29, 2.42, 4.70, and 10.1 mg XDE-208/L and ranged from 94% to 101% of the nominal concentrations. Measured concentrations of XDE-208 in the test-substance treatments on days 7 through 38 ranged from 78 to 117% of the nominal

concentrations. The mean measured test concentrations of XDE-208 in the test-substance treatments for the 38-day exposure were 0.581, 1.21, 2.37, 5.02, and 9.89 mg XDE-208/L and ranged from 89% to 100% of the nominal concentrations. No residues of XDE-208 were detected in the control solution above the MQL of 0.0510 mg XDE-208/L. All biological response results are based upon the mean measured concentrations of XDE-208 during the 30 days of exposure.

The validity criteria in terms of dissolved oxygen concentration, control mortality, hatching success and water temperature were satisfactorily maintained during the test. The concentrations of the test item in solution were satisfactorily maintained within ± 20 % of the mean measured values apart from the test media of nominal 10 mg XDE-208/L. In this concentration level the samples collected at day 3 were outside the demanded range. In one aquaria of this treatment level the concentration was above the nominal concentration and below the nominal concentration in the second aquaria. Since the biological effects in both aquaria were comparable, this short-time incident was considered to be a minor deviation.

Hatching success

Egg hatch began on day 6 in the control and all test substance treatments. Day 0 post-hatch (i.e. ≥95% hatch in the control treatment) was determined to be study day 8. Hatch was completed in the control and all test treatments between study days 8 and 14. Complete hatch (≥95% hatch in the control) was determined to be study day 8 (day 0 post-hatch). All test substance treatments, with the exception of the 0.581 mg XDE-208/L treatment, reached 95% hatch on study day 8. The 0.581 mg XDE-208/L treatment reached 95% hatch on study day 9. Hatch was completed in all treatment replicates between study days 8 and 14, with the exception of the 1.21 mg XDE-208/L treatment. One replicate in the 1.21 mg XDE-208/L treatment did not reach 100% hatch until study day 18 due to one embryo in this replicate not hatching until this day. Overall hatching success in the control was 89%, which met the acceptability criterion for this endpoint. Table B.9.2.1.4 shows the hatching success. There was no statistically significant (p = 0.05) reduction in hatching success or time to start and completion of hatch observed in any of the test substance treatments, as compared to the control.

Table 5.4.1.2.Study 2.1 (DAR Table B.9.2.1.4) Effects of XDE-208 on hatchability, survival and growth of sheepshead minnow

Treatment Expressed as Mean Measured Concentration (mg a.i./L)	No. of Eggs at Study Initiation	% Egg Hatchability	No. of Surviving Fry	% Fry Survival	Mean Length of Surviving Fish (mm)	Mean Wet Weight of Surviving Fish (g)
Negative control	100	89	88	99	14.6	0.0922
0.581	100	88	86	98	14.6	0.0920
1.21	100	93	87	94	14.5	0.0933
2.37	100	93	93	100	14.2*	0.0850
5.02	100	94	92	98	14.1*	0.0844
9.89	101	91	89	97	14.0*	0.0851

* Statistically significant difference (Dunnett's Test; p = 0.05) was observed between the test substance treatment and the control.

	Hatchability	Survival	Growth (based on length)
NOEC (mg a.i.L)	9.89	9.89	1.21
LOEC (mg a.i.L)	>9.89	>9.89	2.37
MATC (mg a.i.L)	Could not be calculated	Could not be calculated	1.69

Post-hatch survival
Post-hatch survival was calculated as the percent of hatched fry that were alive at test termination (study day 38; 30 days post-hatch). Post-hatch survival in the control was 99%, which met the acceptability criterion for this endpoint. Post-hatch survival in the test substance treatments were 98, 94, 100, 98, and 97% in the 0.581, 1.21, 2.37, 5.02, and 9.89 mg XDE-208/L treatments, respectively (Table B.9.2.1.4). There was no statistically significant (p = 0.05) reduction in post-hatch survival observed in any of the test substance treatments, as compared to the control. Table B.9.2.1.4 shows the percentage survival from day 0 until the end of the test.

Weight and length
Growth of surviving fry was assessed at test termination (study day 38; 30 days post-hatch) through standard length and blotted wet weight measurements. The mean weight and length parameters for surviving fry at the end of the test are shown in table B.9.2.1.4. There were statistically significant (p = 0.05) reductions in length of the 2.37, 5.02, and 9.89 mg XDE-208/L test substance treatment fry as compared to the control fry, but no reduction in blotted wet weight in any of the test substance treatments as compared to the control.

Sub-lethal observations
There were no morphological abnormalities observed during the exposure. Some fry (i.e., ≤2% of treatment population) were observed to be laying on the bottom of the chamber, and the fry exhibiting this behavior were present prior to study day 19 in the 1.21, 5.02, and 9.89 mg XDE-208/L treatments. There were no other behavioral abnormalities observed during the exposure. Since swim-up does not occur during the development of sheepshead minnow fry, this endpoint was not part of the behavioral observations and was not part of the statistical evaluations. Sublethal effects of XDE-208 are summarized in Table B.9.2.1.5.

Table 5.4.1.2.Study 2.2 (DAR Study B.9.2.1.5) Sub-lethal effects of XDE-208 on appearance or behavior of sheepshead minnow.

Treatment Expressed as Mean Measured Concentration (mg a.i./L)	Observation Period (Study Day)				
	Fry laying on bottom of test chamber (% affected)				
	Days 9 and 10	Day 11	Days 12 and 13	Day 14	Day 18
Negative control	0	0	0	0	0
0.581	0	0	0	0	0
1.21	2	3	1	0	1
2.37	0	0	0	0	0
5.02	0	0	0	1	0
9.89	0	0	1	0	0

Table 5.4.1.2.Study 2.3 (DAR Study B.9.2.1.6) Summary of the results

Biological Endpoint	No-Observed-Effect Concentration (NOEC)	Lowest-Observed-Effect Concentration (LOEC)	MATC [a]
Egg Hatchability	9.89 mg XDE-208/L	>9.89 mg XDE-208/L	N/C [c]
Fry Survival [b]	9.89 mg XDE-208/L	>9.89 mg XDE-208/L	N/C [c]
Standard Length	1.21 mg XDE-208/L	2.37 mg XDE-208/L	1.69 mg XDE-208/L
Blotted Wet Weight	9.89 mg XDE-208/L	>9.89 mg XDE-208/L	N/C [c]

[a] MATC is the maximum acceptable toxicant concentration, which is calculated as the geometric mean of the NOEC and LOEC values.
[b] Fry survival based on number of hatched fry surviving on day 30 post-hatch.
[c] N/C = could not be calculated.

Reliability of the study
The reported study is GLP compliant and conducted to a standard study protocol without significant deviations. The test results are in compliance with the guideline's validity criteria. It is acceptable for regulatory use.

Since the measured concentrations fall below 80% of the nominal concentrations during the test, the mean measured concentration is used in the risk assessment.

Based on mean measured concentrations, the regulatory endpoint is a 38-day NOEC is 1.21 mg XDE-208/L.

5.4.2 Aquatic invertebrates

Short-term toxicity to aquatic invertebrates

Study 1: Acute toxicity to *Daphnia magna* (Sulfoxaflor DAR, Volume 3 - B.9.2.1.3.i)

Method	Test organism	Test design	Results (mg a.s./L)			Remarks	Reference
			Endpoints	NOEC [mg/L]	LC_{50}/EC_{50} [mg/L]		
OECD 202, OPPTS 850.1010	*Daphnia magna*	acute, 48h, static	immobility	110	>399	mm	Hicks S.L. 2008a

Citation: Hicks S.L. (2008a): Sulfoxaflor: Static Acute Toxicity Test with the Water Flea, *Daphnia magna*. ABC Laboratories, Inc., 7200 E. ABC Lane, Columbia MO 65202, ABC Study Number 63665. Dow AgroSciences unpublished report, Study Number 080068. July 31, 2008.

Guidelines: OECD Guideline 202

OPPTS 850.1010

JMAFF 2-7-2-1

GLP compliance: Yes.

Test material:

Test item: Sulfoxaflor

Purity: 95.6% w/w

Description: White solid

Lot No./Batch No.: E2162-34

Material and methods:

A 48-hour static test was performed with test concentrations of 0 (control), 13, 25, 50, 200, and 400 mg Sulfoxaflor/L. All solution preparations were corrected for the purity of the test substance. A 400 mg Sulfoxaflor/L primary stock solution was prepared at test initiation by diluting 0.8001 g as Sulfoxaflor of the test substance into a 2,000 mL volume of dilution water and sonicating the dilution for approximately 10 minutes. Appropriate volumes of the primary stock solution were used to prepare 1.0 L volumes of the six test substance treatments. The control consisted of dilution water only.

Ten neonates (<24-hours old) were present in each test chamber with two replicates per test treatment, resulting in 20 daphnids per test treatment. The daphnids were observed for immobility and sublethal effects at approximately 24 and 48 hours after test initiation. The test chambers were grouped by treatment in a water bath. No aeration was provided to any test chamber during the test.

Temperature, pH, and dissolved oxygen concentration were measured in each test chamber at test initiation, 24 hours, and at test termination. Alkalinity, hardness, and conductivity were measured in a sample of the dilution water at test initiation.

No statistical analyses were preformed since the percentage of immobile daphnids in the test substance treatments did not exceed 20%.

Results

Water quality parameters (pH, temperature and dissolved oxygen) remained within acceptable testing limits for daphnids throughout the test. The control and test solutions were clear and colourless with no visible signs of undissolved test substance, precipitate, or surface film throughout the study.

Analytical confirmation of the test substance, XDE-208, within the test solutions, was performed at 0 and 48 hours. The measured concentrations in the test substance treatment sample collected at 0 hour were 11.1, 24.8, 52.0, 110, 199, and 393 mg XDE-208/L or 85 to 110% of the nominal concentrations, indicating the treatments were appropriately dosed at test initiation. The measured concentrations in the test substance treatment samples collected at 48 hours were 12.4, 23.7, 50.6,

110, 194, and 405 mg a.i./L or 95 to 110% of the nominal concentrations. The mean measured concentrations in the test solutions were 11.8, 24.3, 51.3, 110, 197, and 399 mg a.i./L or 91 to 110% of the nominal concentrations.

After 48 hours of exposure, immobility was 0, 0, 0, 0, 0, 15, and 20% in the 0 (control), 11.8, 24.3, 51.3, 110, 197, and 399 mg a.i./L treatments (see Table B.9.2.1.7). No subletal effects were observed.

Table 5.4.2.1.Study 1.1 (DAR table B.9.2.1.7) Effect of XDE-208 on immobilization of *Daphnia magna*

Treatment as Mean Measured Concentration (mg a.i./L)	24-hr		48-hr	
	No. Immobile	% immobility	No. Immobile	% immobility
Negative control	0	0	0	0
11.8	0	0	0	0
24.3	0	0	0	0
51.3	0	0	0	0
110	0	0	0	0
197	1	5	3	15
399	1	5	4	20
NOEC	110 mg a.i./L		110 mg a.i./L	
EC50	>399 mg a.i./L		>399 mg a.i./L	

Reliability of the study
The reported study is GLP compliant and conducted to a standard study protocol without significant deviations. The test results are in compliance with the guideline's validity criteria. It is acceptable for regulatory use.

Since the measured concentrations remained between 80 and 120% of the nominal concentrations SANCO/3268/2001 recommends that endpoints should normally be expressed in terms of nominal concentrations. Because the slight differences between nominal and mean measured concentrations will not fundamentally change the toxicity value, the use of toxicity endpoints based on mean measured concentrations is therefore considered to be acceptable for risk assessment purposes.

Based on mean measured concentrations, the regulatory endpoint is a 96-hour EC_{50} >399 mg XDE-208/L, the highest concentration tested.

Study 2: Acute toxicity to marine or estuarine invertebrate: mysid shrimp (*Americamysis bahia*) (Sulfoxaflor DAR, Volume 3 - B.9.2.1.3.iii)

Method	Test organism	Test design	Results (mg a.s./L)			Remarks	Reference
			Endpoints	NOEC [mg/L]	LC_{50}/EC_{50} [mg/L]		
OPPTS 850.1035, EPA 72-1	Mysid shrimp (*Americamysis bahia*)	acute, 96h, static	mortality subleth. effects	0.389	0.643	mm	Hicks S.L. 2008b

Citation: Hicks S.L. (2008b): Sulfoxaflor: Static Acute Toxicity Test with the Mysid Shrimp, *Americamysis bahia*. ABC Laboratories, Inc., 7200 E. ABC Lane, Columbia MO 65202, ABC Study Number 63666. Dow AgroSciences unpublished report, Study Number 080069. September 4, 2008.

Guidelines: OPPTS 850.1035

FIFRA 72-3

GLP compliance: Yes.

Test material:

Test item: Sulfoxaflor

Purity: 95.6% w/w

Description: White solid

Lot No./Batch No.: E2162-34

Material and methods:

A 96-hour static toxicity test was performed with test concentrations of 0 (control), 0.10, 0.20, 0.40, 0.80, and 1.6 mg Sulfoxaflor/L. A 0.050 mg Sulfoxaflor/L primary stock solution was prepared at test initiation by diluting 0.1047 g Sulfoxaflor into a 2,000 mL volume of dilution water. Appropriate volumes of the primary stock solution were used to prepare 1.0 L volumes of the five test substance treatments. The control consisted of dilution water only. The test chambers were grouped by treatment in a water bath.

Ten mysid shrimp (<24-hours old) were added to each of two test chambers for the dilution water control and each test substance treatment that resulted in 20 mysids per test treatment. Mysids were impartially added to a set of labelled containers with each container representing one treatment replicate. Each container was then randomly assigned to a treatment replicate by random number generator. The individuals within each container were then released from the container into the corresponding test chamber. Observations were made daily (± 1hour of test initiation) for mortality and sublethal effects. Gentle aeration was initiated in the control and test treatment chambers after 72 hours. The mysids were offered brine shrimp *ad libitum* daily.

Temperature, dissolved oxygen, pH, and salinity were measured in the replicate test chambers of all treatments daily during the definitive test. Temperature was also recorded continuously from the waterbath using an electronic data-logging system.

Statistical Analysis: All statistical analyses were performed with SAS software. Estimates of LC50 values and their 95% confidence limits were calculated using the probit method and Spearman-Karber method (trimmed or untrimmed). When the P value for Goodness of Fit was >0.05 and there was no other evidence of questionable convergence, the probit method was selected for reporting. When this criterion was not achieved, the Spearman-Karber method was selected for reporting. The NOEC was determined by using a Fisher's exact test. A Hochberg adjustment was used to control the experiment wise error rate for the Fisher's test at the same alpha level.

Results

Salinity, temperature, and pH remained within acceptable limits throughout the 96-hour definitive test. The control and test solutions were clear and colourless with no visible signs of undissolved test substance, precipitate, or surface film throughout the study.

Analytical confirmation of the test substance, XDE-208, within the test solutions, was performed at 0 and 96 hours. The measured concentrations in the test substance treatment samples collected at 0 hour were 0.0895, 0.192, 0.401, 0.750, and 1.58 mg a.i./L or 90 to 100% of the nominal concentrations, indicating the treatments were appropriately dosed at test initiation. The measured concentrations in the test substance treatment samples collected at 96 hours were 0.0915, 0.186,

0.376, 0.745, and 1.59 mg a.i./L or 92 to 99% of the nominal concentrations. The mean measured concentrations in the test solutions were 0.0910, 0.189, 0.389, 0.748, and 1.59 mg/L or 91 to 99% of the nominal concentrations. No residues of XDE-208 were detected in the control solutions above the MQL of 0.00500 mg a.i./L. Since the measured concentrations approximated the nominal concentrations (i.e., within 80 to 120% of nominal) and were stable, the biological response results were based upon the nominal concentrations and the mean measured concentrations.

After 96 hours of exposure, mortality was 0, 0, 0, 0, 75, and 100% in the 0 (control), 0.10, 0.20, 0.40, 0.80, and 1.6 mg a.i./L nominal treatments (B.9.2.1.10). There were no sublethal effects noted in the control or test substance treatments during the definitive test.

Table 5.4.2.1.Study 2.1 (DAR Table B.9.2.1.10) Effect of XDE-208 on mortality of mysid shrimp

Treatment (mg a.i./L)		24-hr		48-, 72-, and 96-hr	
Nominal	**Mean Measured**	**No. Dead**	**% Mortality**	**No. Dead**	**% Mortality**
Negative Control	<MQL	0	0	0	0
0.10	0.0910	0	0	0	0
0.20	0.189	0	0	0	0
0.40	0.389	0	0	0	0
0.80	0.748	14	70	15	75
1.6	1.59	20	100	20	100
NOEC		0.40 mg a.i./L (nominal) 0.389 mg a.i./L (mean measured)		0.40 mg a.i./L (nominal) 0.389 mg a.i./L (mean measured)	
LC50		0.67 mg a.i./L (nominal) 0.666 mg a.i./L (mean measured)		0.67 mg a.i./L (nominal) 0.643 mg a.i./L (mean measured)	

Reliability of the study

The reported study is GLP compliant and conducted to a standard study protocol without significant deviations. The test results are in compliance with the guideline's validity criteria. It is acceptable for regulatory use.

Since the measured concentrations remained between 80 and 120% of the nominal concentrations SANCO/3268/2001 recommends that endpoints should normally be expressed in terms of nominal concentrations. Because the slight differences between nominal and mean measured concentrations will not fundamentally change the toxicity value, the use of toxicity endpoints based on mean measured concentrations is therefore considered to be acceptable for risk assessment purposes.

Based on mean measured concentrations, the regulatory endpoint is a 96-hour LC_{50} 0.643 mg XDE-208/L.

Long-term toxicity to aquatic invertebrates

Study 3: Chronic toxicity to *Daphnia magna* (Sulfoxaflor DAR, Volume 3 - B.9.2.1.3.ii)

Method	Test organism	Test design	Results (mg a.s./L)			Remarks	Reference
			Endpoints	NOEC [mg/L]	LC_{50}/EC_{50} [mg/L]		
OECD 211, OPPTS 850.1300	*Daphnia magna*	chronic, 21d, semi-static	mortality reproduction growth	50	-	nom	Kuhl, R, Wydra, V. 2009a

Citation: Kuhl, R, Wydra, V. (2009a): Sulfoxaflor technical: Influence of Sulfoxaflor technical to *Daphnia magna* in a Reproduction Test. IBACON GmbH, Arheiliger Weg 17, 64380 Rossdorf, Germany, Laboratory Project Number: 46842221. Dow AgroSciences unpublished report, Study Number 080445. Study Report Completion Date: May 07, 2009

Guidelines: OECD Guideline 211, 2008

OPPTS 850.1300, 1996

JMAFF 2-7-2-3, 2000

GLP compliance: Yes.

Test material:

Test item: Sulfoxaflor

Purity: 95.6% w/w

Description: White solid

Lot No./Batch No.: E2162-34

Material and methods:

In a 21-day semi-static test, young daphnids were exposed to the test item Sulfoxaflor technical at the nominal concentrations of 100, 50, 25, 12.5 and 6.25 mg Sulfoxaflor/L. The test media were renewed on Days 3, 5, 7, 10, 12, 14, 17 and 19 of the exposure period (three times a week). Before test start and before the test medium renewal, the test medium of the highest test concentration of nominal 100 mg/L was prepared by dissolving 200 mg test item into 2000 mL culture medium by intense stirring for 15 minutes and short ultrasonic treatment for 10 minutes. Adequate volumes of this test medium were diluted with culture medium. The test media were prepared just before introduction of daphnids (= start of the test and each test medium renewal).

One neonate (<24-hours old) was present in each test chamber with ten replicates per test treatment, resulting in 10 daphnids per test treatment. The test chambers were grouped by treatment in a water bath. No aeration was provided to any test chamber during the test. The mortality of the test animals and the number of young animals were recorded each day. At the end of the study the body length excluding the anal spine of each surviving adult daphnid was measured using a digital camera and a program for digital length measurement.

The pH, temperature and dissolved oxygen were measured in the all test treatments and in the control at the start and end of each exposure period.

Statistical analysis: The NOEC and the LOEC for the reproduction rate and the length evaluated by the Dunnett´s Multiple T-test after analysis of variance (ANOVA). The NOEC and the LOEC for days to first brood were evaluated by the Student T-test after analysis of variance (ANOVA). The EC50 (21 d) of the reproduction rate was determined by probit analysis.

Deviations to the study plan:

In the aged test medium of the highest concentration at Day 14, the temperature was 22ºC. All other temperatures were 20ºC ± 1ºC.

Presumed effect on the study: None, since it was only a slight deviation and survival and reproduction seem not to be affected.

Results
Water quality parameters (pH, temperature and dissolved oxygen) remained within acceptable testing limits for daphnids throughout the test. The control and test solutions were clear and colourless with no visible signs of undissolved test substance, precipitate, or surface film throughout the study.

Analytical samples were collected from all freshly prepared and aged test media. The concentrations of the test item XDE-208 technical were measured in the duplicate test medium samples from all test concentrations at days 0, 7, 14 (freshly prepared test media) and at days 3, 10, 17 (aged test media after 72 hours of exposure). From the control samples only one of the duplicate samples was analyzed. Summary of the analytical results is given in Table B.9.2.1.8. At the start of the test just before introduction of daphnids 101% of the nominal test concentrations were found. After 72 hours test duration 101% of the nominal values were determined. Thus, during the test period of 72 hours daphnids were exposed to a mean of 101 % of nominal. Since 72 hours is the longest renewal period during the test, it can be assumed that the test item was also stable during the further non-measured renewal periods of 48 hours. Therefore, all reported results are related to nominal concentrations of the test item.

Table 5.4.2.Study 3.1 (DAR Table B.9.2.1.8) Summary of analytical results

sample description [mg/L]	% of nominal[1]	n
control	n.a.	6
6.25	100	12
12.5	101	12
25	101	12
50	101	12
100	101	12

[1] mean value of all measured samples per treatment group
n number of analysed samples
n.a. not applicable

Effects of XDE-208 on survival, growth and reproduction of *Daphnia* are summarized in Table B.9.2.1.9. No mortality occured in the control and any treatment groups during the test. No sublethal effects in the control and any treatment groups were observed during the test.

Table 5.4.2.Study 3.2 (DAR Table B.9.2.1.9) Effects of XDE-208 on survival, growth and reproduction of *Daphnia*

Treatment (mg a.i./L)	Day 0 to 21	At test termination			
	Survival of adult daphnids (%)	Mean days to first brood	Total No. of live offspring produced	Mean number of live offspring produced per surviving daphnid	Mean length of surviving adults (mm)
control	10	10.8	711	71.1	4.3
6.25	10	11.2	832	83.2	4.3
12.5	10	11.9	717	71.7	4.3

Treatment (mg a.i./L)	Day 0 to 21	At test termination			
	Survival of adult daphnids (%)	Mean days to first brood	Total No. of live offspring produced	Mean number of live offspring produced per surviving daphnid	Mean length of surviving adults (mm)
25	10	11.7	566	56.6	4.3
50	10	12.1	602	60.2	4.0
100	10	14.6	426	42.6	4.2
	Survival	Reproduction			Growth
EC_{50} (mg a.i./L)	> 100	> 100			> 100
NOEC (mg a.i./L)	≥ 100	50			≥ 100
LOEC (mg a.i./L)	> 100	100			> 100

Reliability of the study
The reported study is GLP compliant and conducted to a standard study protocol with a minor deviation that had no significant effect on the study. The test results are in compliance with the guideline's validity criteria. It is acceptable for regulatory use.

The regulatory endpoint is a 21-day NOEC 50 mg XDE-208/L (based reproduction and nominal concentrations).

Study 4: Chronic toxicity to marine or estuarine invertebrate: mysid shrimp (*Americamysis bahia*) (Sulfoxaflor DAR, Volume 3 - B.9.2.1.3.iv)

Method	Test organism	Test design	Results (mg a.s./L)			Remarks	Reference
			Endpoints	NOEC [mg/L]	LC_{50}/EC_{50} [mg/L]		
OPPTS 850.1350, 850.1000 EPA 72-3	Mysid shrimp (*Americamysis bahia*)	chronic, 28d flow-through	mortality length subleth. effects	0.114	-	mm	Lehman, Ch. 2010

Citation: Lehman, Ch. (2010): Sulfoxaflor: Life-Cycle Toxicity Test of the Saltwater Mysid, *Americamysis bahia*, Conducted under Flow-Through Conditions. ABC Laboratories, 7200 E. ABC Lane, Columbia, Missouri 65202, ABC Laboratories Project Number 65177. Dow AgroSciences unpublished report, Study Number 090534. April 29, 2010.

Guidelines: OPPTS 850.1350 and 850.1000

FIFRA 72-3

GLP compliance: Yes.

Test material:

Test item: Sulfoxaflor

Purity: 95.6% w/w

Description: White solid

Lot No./Batch No.: TSN003725-0001, E2162-34

Material and methods:

A 28-day flow through toxicity test was performed with test concentrations of 0 (control), 0.063, 0.13, 0.25, 0.50, and 1.0 mg Sulfoxaflor/L Diluter stock solutions were prepared at a target concentration of 286 mg Sulfoxaflor /L at least once every three to six days by diluting approximately 5.3849 g of Sulfoxaflor per 18 L dilution water. An FMI metering pump introduced 40-mL volumes of the diluter stock solution to the diluter system, where the diluter stock solution volume was diluted with approximately 285.825 mL of dilution water. The usage of the Sulfoxaflor stock solution was monitored and recorded daily. Proper function of the injector was verified twice each day during the exposure. During the course of the definitive test, approximately 92.7 L of dilution-water control and test solution were delivered to each chamber each day while only one side of the test chamber was in use. This rate was sufficient to provide approximately 5.8 volume additions in a 24-hour period.

Mysids <24 hours old were impartially added to a set of labelled plastic containers prior to their distribution into the test chambers at test initiation. To accurately count the mysids, it was necessary to use three plastic containers, each containing five mysids for a total of 15 mysids for the three containers. Each set of containers was labelled numerically and randomly assigned to a treatment replicate and retention basket by a computer-generated random number table. Each retention basket received 15 mysids, for a total of 30 mysids per control treatment or test substance treatment replicate and a total of 90 mysids per control treatment or test-substance treatment. Mysids were fed *ad libitum* brine shrimp nauplii (*Artemia* sp.; 24-48 hours old) at least two times daily. Observations of mortality and sublethal responses F_0-mysid generation were made daily for the duration of the testing period. The number of females with brood pouches was enumerated from the time brood pouches were first noted (day 10) until adults were paired on day 13. The body lengths of mysids (as measured by total midline body length) were measured to the nearest 0.1 mm with a dissecting microscope. Ovigerous F_0-female mysids (i.e., females with eggs within the marsupium) were isolated and paired with adult males and transferred to the brood baskets on day 13 of the exposure. Once paired, the mysids in the brood cups were observed for mortality and reproduction (i.e., young per female). The first day young were observed was considered the day of first brood, although release of these young may have occurred over 2 or 3 days. After 14 days of exposure, the body length of all surviving F_0 mysids present in the growth-retention basket was measured. The growth-retention baskets were terminated following these measurements. The F_1-mysid exposure phase of the test was initiated with the first 15 post-larval F_1 mysids, or fewer when 15 young were not available. The post-larval F_1 mysids were assigned to retention baskets within the same test chambers as the F_0-mysid exposure. The isolated F_1 mysids were observed daily for mortality during the exposure and when F_1 mysids were 10 days old. The F_1 mysids were terminated when they reached 10 days of age because this was the maximum achievable age for all F_1 mysids at termination of the F_0-mysid exposure (i.e., study day 28). The body length of all surviving 10-day old F_1 mysids was measured.

Temperature, dissolved oxygen, and pH were measured in the replicate test chambers of all treatments at test initiation and termination and at least weekly during the definitive test. Test solution salinity was measured daily in all control and treatment replicate test chambers and light intensity was measured on day 28.

Statistical Analysis: All statistical analyses were performed using SAS software. The NOECs, based on percent survival, survival of second generation offspring, reproduction (i.e., young per female), and adult length, were estimated using a one-way analysis of variance (ANOVA) procedure and either one-tailed Fisher's test with Hochberg’s family wise adjustment for significance or a one-tailed Dunnett's test. The alternate hypothesis is that the mean for the parameter in the treated

exposures was reduced in comparison to the negative control mean for that same parameter. The time to first brood release was analyzed using a one-tailed Dunnett's test to determine significant inhibiting or enhancing effects on this parameter. For all analyses, prior to the Dunnett's test, a Shapiro-Wilk test for normality and Levene's test for homogeneity of variance over treatments were conducted at each time point. The assumptions of normality and homogeneity of variance were not met for the raw or transformed day 14 body length data and the day 28 survival data; therefore, a nonparametric analysis was performed on the ranks of the data. All other parameters met the assumptions of normality and homogeneity of variance and were analyzed with a parametric ANOVA. The maximum acceptable toxicant concentration (MATC) was calculated as the square root of the product of the NOEC and LOEC concentrations for the most sensitive toxicological endpoint. Median lethal concentration (LC50) values were calculated from the 7-, 14-, 21-, and 28-day data sets. Estimates of LC50 values and their 95% confidence limits were calculated using the probit method and Trimmed Spearman-Karber method.

Results

All measured water-quality parameters during the 28-day exposure were within the limits specified by the study protocol. The control and test solutions were clear and colourless with no visible signs of undissolved test substance, precipitate, or surface film throughout the study.

During the definitive test, the concentrations of XDE-208 in test solutions were determined in samples collected prior to initiation (day –N) and on study days 0, 7, 14, 21, and 28 (termination) of the definitive test. Samples were vialed and analyzed using LC-MS/MS. Measured concentrations of XDE-208 in the test-substance treatments prior to initiation of the definitive test were 0.0582, 0.108, 0.228, 0.416, and 0.905 mg XDE-208/L and ranged from 83% to 91% of the nominal concentrations. Measured concentrations of XDE-208 in the test-substance treatments on day 0 of the exposure were 0.0566, 0.110, 0.236, 0.458, and 0.925 mg XDE-208/L and ranged from 85% to 94% of the nominal concentrations. Measured concentrations of XDE-208 in the test-substance treatments on days 7 through 28 ranged from 87 to 99% of the nominal concentrations. Analytical measurements of the level 5 (1.0 mg XDE-208/L) test solutions were not performed on days 14, 21, and 28, due to 100% mortality at that level. The mean measured test concentrations of XDE-208 in the test substance treatments for the 28-day exposure were 0.0603, 0.114, 0.239, 0.470, and 0.918 mg XDE-208/L and ranged from 88% to 96% of the nominal concentrations. The measured concentration of the diluter stock solutions ranged from 93% to 105% of the nominal concentrations during the exposure period. All biological response results are based upon the nominal and mean measured concentrations of XDE-208 during the 28 days of exposure.

Effects of XDE-208 on survival, growth and reproduction of mysid shrimp are given in Table B.9.2.1.11. Sub-lethal effects of XDE-208 on appearance or behavior in mysid shrimp are shown in Table B.9.2.1.12.

Table 5.4.2.Study 4.1 (DAR Table B.9.2.1.11) Effects of XDE-208 on survival, growth and reproduction of mysid shrimp.

Treatment (mg a.i./L)	Day 0 to 13	Day 13 to 28	At Test Termination				
	% Mortality of First Generation Mysids Before Pairing	% Mortality of First Generation Mysids After Pairing	Total No. of Live Offspring	Mean No. of Live Offspring per Female	Mean Days to First Brood	Mean Length of Surviving Adults (mm)*	
						Male	Female
Negative control	0	2	470	22.4	17.8	6.08	6.36
0.063	7	0	599	30.2	17.6	5.97	6.24
0.13	2	9	604	28.8	17.5	6.05	6.22

Treatment (mg a.i./L)	Day 0 to 13	Day 13 to 28	At Test Termination				
	% Mortality of First Generation Mysids Before Pairing	% Mortality of First Generation Mysids After Pairing	Total No. of Live Offspring	Mean No. of Live Offspring per Female	Mean Days to First Brood	Mean Length of Surviving Adults (mm)*	
						Male	Female
0.25	2	9	652	31.0	17.0**	5.98	6.30
0.50	5	4	608	29.0	17.0**	5.69**	6.31
1.0	100*	100*	---	---	---		

* Indicates a statistically significant difference (Fisher's Exact Test; $p \leq 0.05$) as compared to the negative control.
** Statistically significant differences (Dunnett's Test; $p \leq 0.05$) as compared to the negative control.

Nominal concentrations	Survival	Reproduction		Growth	
LC_{50} (mg a.i./L)	0.633 (95% CL: 0.583 and 0.687)	NA	NA	NA	
NOEC (mg a.i./L)	0.50	0.50	0.13	0.25	0.50
LOEC (mg a.i./L)	1.0	> 0.50	0.25	0.50	>0.50
MATC (mg a.i./L)	0.71	NA	0.18	0.35	NA
Mean measured concentrations	Survival	Reproduction		Growth	
LC_{50} (mg a.i./L)	0.587 (95% CL: 0.540 and 0.638)	NA	NA	NA	
NOEC (mg a.i./L)	0.470	0.470	0.114	0.239	0.470
LOEC (mg a.i./L)	0.918	> 0.470	0.239	0.470	>0.470
MATC (mg a.i./L)	0.657	NA	0.165	0.335	NA

Table 5.4.2.Study 4.2 (DAR Table B.9.2.1.12) Sub-lethal effects of XDE-208 on appearance or behavior in mysid shrimp

Treatment (mg a.i./L)	Observation period			
	Observation - Days 7 and 14 (prior to pairing) (% affected)		Observation 2 – Day 21 and 28 (after pairing) (% affected)	
	Day 7	Day 13	Day 21	Day 28
Negative control	0	0	0	0
0.063	0	0	0	0
0.13	0	0	0	0
0.25	0	0	0	0
0.50	0	0	0	0
1.0	0	0	0	0

Note: There were no sublethal effects of XDE-208 on appearance or behavior observed during the study.

Reliability of the study

The reported study is GLP compliant and conducted to a standard study protocol without significant deviations. The test results are in compliance with the guideline's validity criteria. It is acceptable for regulatory use.

Since the measured concentrations remained between 80 and 120% of the nominal concentrations SANCO/3268/2001 recommends that endpoints should normally be expressed in terms of nominal

concentrations. Because the slight differences between nominal and mean measured concentrations will not fundamentally change the toxicity value, the use of toxicity endpoints based on mean measured concentrations is therefore considered to be acceptable for risk assessment purposes.

Based on mean measured concentrations, the regulatory endpoint is a 28-day NOEC 0.114 mg XDE-208/L.

5.4.3 Algae and aquatic plants

Study 1: Toxicity to the green alga *Pseudokirchneriella subcapitata* (Sulfoxaflor DAR, Volume 3 - B.9.2.1.4.i)

Method	Test organism	Test design	Results (mg a.s./L)			Remarks	Reference
			Endpoints	NOEC [mg/L]	LC_{50}/EC_{50} [mg/L]		
OECD 201, OPPTS 850.5400	Freshwater green (*Pseudokirchneriella subcapitata*)	growth inhibition, 96h, static	biomass yield growth rate	100	>100 >100 >100	nom	Dengler, D. 2009a

Citation: Dengler, D. (2009a) Sulfoxaflor: Testing of Effects of Sulfoxaflor on the Single Cell Green Alga *Pseudokirchneriella subcapitata* in a 96 h Static Test. Eurofins-GAB GmbH, Eutinger Str. 24, D-75223 Niefern-Öschelbronn, Germany, Study code: S08-03025 Dow AgroSciences unpublished report, Study Number 080439. Study Report Completion Date 27 July 2009

Guidelines: OECD Guideline 201

OPPTS 850.5400 (1996)

GLP compliance: Yes.

Test material:

Test item: Sulfoxaflor

Purity: 95.6% w/w

Description: White solid

Lot No./Batch No.: E2162-34

Material and methods:

A 96-hour static test was conducted. Because of no effects in a range-finding test the definitive test was performed in a limit test design. The algae were exposed to the control and one concentration of Sulfoxaflor (100 mg a.s./L) under defined conditions in a synthetic growth medium during several generations. Controls and 100 mg/L were tested in six replicates. The necessary amount of test item for preparing the stock solution S1 was weighed on weighing scoops and transferred to a volumetric flask 1000 mL with approximately 500 mL medium. This solution was homogenised by ultrasonic dispersion. Then, algae suspension and test medium were added up to the bench mark (see Table 3). This solution was distributed into the test vessels. The test solution volume was 167 mL per test vessel.

By comparing the cell division under test conditions with and without the influence of test item, the

inhibitory effect (EC, effect concentration) on the cell multiplication was calculated. After 1, 2, 3 and 4 days of growth, the cell numbers were measured by fluorescence detection, and the influence on growth was determined.

Statistical analysis: To estimate the LOEC, and hence the NOEC, ANOVA was used to calculate the mean average specific growth rate. The resulting mean for the test item was compared with the control mean (all controls pooled) using the comparison method of Dunnett's test. A test for normality of the data was done by calculating the Shapiro-Wilk's statistic.

Deviations to the study plan:

1. The final composition of the test medium was 1.5 fold of the initial nutrient concentrations.
   Presumed effect on the study: None.

2. Keeping of Stock Cultures: Temperature was 23 ± 2 °C instead of 24 ± 2 °C for technical reasons.
   Presumed effect on the study: None.

Results
Test conditions (light intensity, temperature and pH) remained within acceptable testing limits for algae.

The concentration course of XDE-208 was verified in test medium by analysing the contents in the samples over the whole test period in intervals of 24 hours. Samples were taken after initiation of the test and thereafter in 1 d intervals until the end of the test after 96 h at the concentration levels of 100 mg/L and control. The concentration courses can be seen in Table B.9.2.1.29. Analytical confirmation of concentrations confirmed that XDE-208 was correctly administered to the test vessels and was stable in the test medium. The toxicological endpoints, therefore, were based on the nominal test item concentrations.

Table 5.4.3.Study 1.1 (DAR Table B.9.2.1.29) Summary of analytical results

Time [h]	nominal concentration of test item [mg/L]		
	0	100	
	nominal concentration of XDE-208 [mg/L]		
	0	95.60	
	actual concentration of XDE-208		
		in mg/L in %	in mg/L in %
0	< LOQ	99.68	104
24	< LOQ	100.11	105
48	< LOQ	103.43	108
72	< LOQ	101.84	107
96	< LOQ	100.37	105
Mean	-	101.09	106

The average cell numbers for each concentration and time of sampling are shown in Table B.9.2.1.29. The percentage inhibition of average specific growth rate, yield, and biomass integral, calculated for t = 96 h, is presented in Table B.9.2.1.30. The results were checked by Dunnett's t test and no significant differences from controls were found.

Table 5.4.3.Study 1.2 (DAR Table B.9.2.1.29) Average cell number for each sampling time and concentration

XDE-208 [mg/L]	Average cell numbers/mL (x 10^4)				
	0 h	24 h	48 h	72 h	96 h
0	0.75	2.57	7.78	30.64	60.32
100	0.75	2.41	6.69	31.77	58.96

Table 5.4.3.Study 1.3 (DAR Table B.9.2.1.30) Percent inhibition of growth rate, yield and biomass after 72 hours

XDE-208 [mg/L]	% Inhibition of growth rate (0 – 96 h)	% Inhibition of yield (96 h)	% Inhibition of biomass integral (0 – 96 h)
0	0	0	0
100	0.6	2.3	1.2

Effects of XDE-208 on algal growth of *Pseudokirchneriella subcapitata* are sumarized in Table B.9.2.1.31.

Table 5.4.3.Study 1.4 (DAR Table B.9.2.1.31) Effects of XDE-208 on algal growth of *Pseudokirchneriella subcapitata*

Hour	EC Type	EC Value [mg a.i./L]	95% Confidence Limits [mg a.i./L]	LOEC [mg a.i./L]	NOEC [mg a.i./L]
96	E_rC_{50} (growth rate)	> 100 (nominal)	-	> 100 (nominal)	100 (nominal)
	E_yC_{50} (yield)				
	E_bC_{50} (biomass)				

Reliability of the study

The reported study is GLP compliant and conducted to a standard study protocol. Two deviations to the study plan were noted, but they are considered minor and they are not supposed to result in any significant effects on the study. The test results are in compliance with the guideline's validity criteria. The study is acceptable for regulatory use.

Since the measured concentrations remained between 80 and 120% of the nominal concentrations SANCO/3268/2001 recommends that endpoints should normally be expressed in terms of nominal concentrations. Because the slight differences between nominal and mean measured concentrations will not fundamentally change the toxicity value, the use of toxicity endpoints based on mean measured concentrations is therefore considered to be acceptable for risk assessment purposes.

The regulatory endpoints are 96-hour E_bC_{50}, E_rC_{50} and E_yC_{50} > 100 mg XDE-208/L (based on nominal concentrations).

Study 2: Toxicity to the saltwater diatom (*Skeletonema costatum*) (Sulfoxaflor DAR, Volume 3 - B.9.2.1.4.ii)

Method	Test organism	Test design	Results (mg a.s./L)			Remarks	Reference
			Endpoints	NOEC [mg/L]	LC_{50}/EC_{50} [mg/L]		
OECD 201, OPPTS 850.5400	Saltwater diatom (*Skeletonema costatum*)	growth inhibition, 96h, static	biomass yield growth rate	109	>109 >109 >109	mm	Dengler, D. 2009b

Citation: Dengler, D. (2009b): Testing of Effects of Sulfoxaflor on the Marine Diatom *Skeletonema costatum* in a Static 96 h Test. Eurofins-GAB GmbH, Eutinger Str. 24, D-75223 Niefern-Öschelbronn, Germany, Phone: 0049(0)7233 96 27 49, Fax: 0049(0)7233 96 27 68, Study code: S08-03027 Dow AgroSciences unpublished report, Study Number 080440, 30 September 2009

Guidelines: OECD Guideline 201

OPPTS 850.5400 (1996)

GLP compliance: Yes.

Test material:

Test item: Sulfoxaflor

Purity: 95.6% w/w

Description: White solid

Lot No./Batch No.: E2162-34

Material and methods:

A 96-hour static test was performed with concentrations of 0 (control), 3.13, 6.25, 12.5, 25, 50 and 100 mg/L. The controls were made in six replicates, the test item flasks were prepared in triplicate. The test flasks were inoculated with cells from exponentially growing cultures to an initial cell density of $1 \cdot 10^4$ cells/mL. The necessary amounts of test item for preparing the stock solutions S1, S2 and S3 (100, 50 and 25 mg/L) were weighed on weighing scoops and transferred to algae medium in volumetric flasks of 500 mL and 1000 mL, respectively. These solutions were homogenised by ultrasonic dispersion. Then, algae suspension and test medium were added up to the bench mark. Solution S3 was diluted accordingly to give the final test concentrations 12.5, 6.25 and 3.13 mg/L (S4, S5, S6). The test solution volume was 167 mL per test vessel.

By comparing the cell division under test conditions with and without the influence of test item, the inhibitory effect (EC, effect concentration) on the cell multiplication was calculated. After 1, 2, 3 and 4 days of growth, the cell numbers were determined by counting, and the influence on growth was determined.

Statistical analysis: To estimate the LOEC, and hence the NOEC for each of the three growth indices (specific growth rate, algal yield and biomass integral), the mean value for each growth descriptor at each test concentration was compared with the corresponding control mean (all six control replicates pooled), using the multiple Dunnett's test. The Shapiro-Wilk's test was used to test the normality of the data. After confirming that the percent inhibition data were normally distributed, the EC50 values for each of the three growth indices were calculated by probit analysis.

Deviations to the study plan:

Cell numbers were determined by counting, not by fluorescence detection.

Reason: No method established.

Impact on study: None.

Results

Test conditions (light intensity, temperature and pH) remained within acceptable testing limits for algae.

Samples were analysed from the inoculated test medium at t = 0 and 4 days. The concentration course of XDE-208 was verified in test medium by analysing the contents at the beginning and at the end of the test (4 d). The concentration courses can be seen in Table B.9.2.1.32. The mean concentration of XDE-208 was 109 % of nominal at the measured concentration levels. The test item was stable during the entire test period.

Table 5.4.3.Study 2.1 (DAR Table B.9.2.1.32) Summary of analytical results

Test item nominal [mg/L]	XDE-208 nominal [mg/L]	Time [d]	XDE-208		
			mg/L	% of nominal	% mean
0	0	0 4	n.d. n.d.	- -	-
100	95.60	0 4	101 106	106 111	109

n.d.: not detectable - not calculated

The average cell numbers for each concentration and time of sampling are shown in Table B.9.2.1.33. The percentage inhibition of average specific growth rate, yield, and biomass integral, calculated for t = 4 d, is presented in Table B.9.2.1.34. The nominal exposure concentrations were used in the result calculation. The results were checked by Dunnett's t-test and no significant differences from controls were found.

Table 5.4.3.Study 2.2 (DAR Table B.9.2.1.33) Average cell number for each sampling time and concentration

XDE-208 [mg/L]	Average cell numbers/mL *				
	0 d	1 d	2 d	3 d	4 d
0	1.00	5.58	27.33	74.14	89.82
100	1.00	4.99	24.26	86.00	103.97

* Algae counts are divided by 10000. At the start, the cell density was adjusted to $1.0 \cdot 10^4$ cells/mL

Table 5.4.3.Study 2.3 (DAR Table B.9.2.1.34) Percent inhibition of growth rate, yield and biomass after 4 days

XDE-208 [mg/L]	% Inhibition of growth rate (0 – 4 d)	% Inhibition of yield (4 d)	% Inhibition of biomass integral (0 – 4 d)
0	0.0	0	0
100	-3.2	-15.9	-10.3

Effects of XDE-208 on algal growth of *Skeletonema costatum* are sumarized in Table 5.4.3.Study 2.4 (DAR Table B.9.2.1.35):

Table 5.4.3.Study 2.4 (DAR Table B.9.2.1.35) Effects of XDE-208 on algal growth of *Skeletonema costatum*

Hour	EC Type	EC Value [mg a.i./L]	95% Confidence Limits [mg a.i./L]	LOEC [mg a.i./L]	NOEC [mg a.i./L]
96	E_rC_{50} (growth rate)	> 100 (nominal) > 109 (mean measured)	-	> 100 (nominal) > 109 (mean measured)	100 (nominal) 109 (mean measured)
	E_yC_{50} (yield)				
	E_bC_{50} (biomass)				

Reliability of the study

The reported study is GLP compliant and conducted to a standard study protocol with a minor deviation to the study plan that is considered minor and is not supposed to result in any significant effects on the study. The test results are in compliance with the guideline's validity criteria. The study is acceptable for regulatory use.

Since the measured concentrations remained between 80 and 120% of the nominal concentrations SANCO/3268/2001 recommends that endpoints should normally be expressed in terms of nominal concentrations. Because the slight differences between nominal and mean measured concentrations will not fundamentally change the toxicity value, the use of toxicity endpoints based on mean measured concentrations is therefore considered to be acceptable for risk assessment purposes.

The regulatory endpoints are 96-hour E_rC_{50}, E_yC_{50} and E_bC_{50} >109 mg XDE-208/L and NOEC 109 mg XDE-208/L (based on mean measured concentrations), the highest concentration tested.

TABLE 5.4.3.STUDY 2.5 (DAR, Table - B.9.2.1.4.iii) Toxicity to the Cyanobacteria (*Anabaena flos-aquae*)

Method	Test organism	Test design	Results (mg a.s./L)			Remarks	Reference
			Endpoints	NOEC [mg/L]	LC_{50}/EC_{50} [mg/L]		
OECD 201, OPPTS 850.5400	Freshwater cyanobacteria (*Anabaena flos-aquae*)	growth inhibition, 96h, static	biomass yield growth rate	13	>98.3 >91.2 >104	mm	Dengler, D. 2009c

Citation: Dengler, D. (2009c) Sulfoxaflor: Testing of Effects of Sulfoxaflor on the Blue Green Alga *Anabaena flos-aquae* in a 96 h Static Test. Eurofins-GAB GmbH, Eutinger Str. 24, D-75223 Niefern-Öschelbronn, Germany, Study code: S08-03028 Dow AgroSciences unpublished report, Study Number 080442. 31 August 2009

Guidelines: OECD Guideline 201

OPPTS 850.5400 (1996)

GLP compliance: Yes.

Test material:

Test item: Sulfoxaflor

Purity: 95.6% w/w

Description: White solid

Lot No./Batch No.: E2162-34

Material and methods:

A 96-hour static test was performed with concentrations of 0 (control), 3.13, 6.25, 12.5, 25, 50 and 100 mg/L. The controls were made in six replicates, the test item flasks were prepared in triplicate. The test flasks were inoculated with cells from exponentially growing cultures to an initial cell density of $1 \cdot 10^4$ cells/mL. The necessary amounts of test item for preparing the stock solutions S1, S2 and S3 (100, 50 and 25 mg/L) were weighed on weighing scoops and transferred to algae medium in volumetric flasks of 500 mL and 1000 mL, respectively. These solutions were homogenised by ultrasonic dispersion. Then, algae suspension and test medium were added up to the bench mark. Solution S3 was diluted accordingly to give the final test concentrations 12.5, 6.25 and 3.13 mg/L (S4, S5, S6). The test solution volume was 167 mL per test vessel.

By comparing the cell division under test conditions with and without the influence of test item, the inhibitory effect (EC, effect concentration) on the cell multiplication was calculated. After 1, 2, 3 and 4 days of growth, the cell numbers were measured by fluorescence detection, and the influence on growth was determined.

Statistical analysis: To estimate the LOEC, and hence the NOEC for each of the three growth indices (specific growth rate, algal yield and biomass integral), the mean value for each growth descriptor at each test concentration was compared with the corresponding control mean (all six control replicates pooled), using the multiple Dunnett's test. The Shapiro-Wilk's test was used to test the normality of the data. After confirming that the percent inhibition data were normally distributed, the EC50 values for each of the three growth indices were calculated by probit analysis.

Deviations to the study plan:

1. Test Organism: Another *Anabaena* strain was used (UTEX LB 2558).
   Reason: Better growth characteristics.

   Impact on study: None.

2. Test Medium: Medium following SCHLÖSSER (1994) was used instead of medium following RIPPKA & HERDMAN (1992).
   Reason: Recommended by the Umweltbundesamt.

   Impact on study: None.

3. Test conditions: Maximum light intensity was > 2150 lux.

   Reason: Technical reason.

   Impact on study: None.

Results
Test conditions (light intensity, temperature and pH) remained within acceptable testing limits for algae.

The concentration course of XDE-208 was verified in test medium by analysing the contents at the beginning and at the end of the test (96 h). Samples were taken from 3.13, 12.5 and 100 mg/L and

control. The concentration courses can be seen in Table B.9.2.1.36. The mean test concentrations were 104 % of nominal. The test item was stable throughout the entire test period. The toxicological evaluation, therefore, was done with nominal test concentrations and calculated mean measured concentrations determined by multiplying the overal mean recovery of 104% by the nominal concentrations.

Table 5.4.3.Study 3.1 (DAR Table B.9.2.1.36) Summary of analytical results

Test item nominal [mg/L]	XDE-208 nominal [mg/L]	Time [d]	XDE-208		
			mg/L	% of nominal	% mean
0	0	0 96	n.d. n.d.	- -	-
3.13	2.99	0 96	3.06 3.06	102 102	102
12.5	11.95	0 96	12.1 12.6	101 105	103
100	95.60	0 96	101 106	106 111	109

n.d.: not detectable - not calculated

The average cell numbers for each concentration and time of sampling are shown in Table B.9.2.1.37. The percentage inhibition of average specific growth rate, yield, and biomass integral, calculated for t = 4 d, is presented in Tables B.9.2.1.38-40. The nominal exposure concentrations were used in the result calculation. The results were checked by Dunnett's t-test.

Table 5.4.3.Study 3.2 (DAR Table B.9.2.1.37) Average cell number for each sampling time and concentration

XDE-208 [mg/L]	Average cell numbers/mL *				
	0 d	1 d	2 d	3 d	4 d
0	1.00	5.80	26.54	111.05	141.94
3.13	1.00	5.97	24.92	107.27	87.41
6.25	1.00	4.85	25.16	120.21	85.07
12.5	1.00	5.26	23.66	109.91	71.41
25	1.00	5.66	21.02	88.95	69.66
50	1.00	4.34	17.06	68.47	57.94
100	1.00	4.24	13.16	52.45	40.03

* Algae counts are divided by 10000. At the start, the cell density was adjusted to $1.0 \cdot 10^4$ cells/mL

Table 5.4.3.Study 3.3 (DAR Table B.9.2.1.38) Percent inhibition of growth rate after 1, 2, 3 and 4 days

XDE-208 [mg/L]	1 d	2 d	3 d	4 d
0	0	0	0	0
3.13	-1.7	1.9	0.7	9.8*
6.25	10.2*	1.6	-1.7	10.3*
12.5	5.5*	3.5*	0.2	13.8*
25	1.4	7.2*	4.7*	14.4*
50	16.5*	13.5*	10.3*	18.1*
100	17.8*	21.4*	15.9*	25.5*

* significant differences from control (Dunnett's test, $p \leq 0.05$)
Negative values mean growth promotion effects

Table 5.4.3.Study 3.4 (DAR Table B.9.2.1.39) Percent inhibition of yield after 1, 2, 3 and 4 days

XDE-208 [mg/L]	1 d	2 d	3 d	4 d
0	0	0	0	0
3.13	-3.5	6.3*	3.4	38.7*
6.25	19.8*	5.4	-8.3	40.4*
12.5	11.3*	11.3*	1.0	50.0*
25	2.9	21.6*	20.1*	51.3*
50	30.4*	37.1*	38.7*	59.6*
100	32.5*	52.4*	53.2*	72.3*

* significant differences from control (Dunnett's test, $p \leq 0.05$)
Negative values mean growth promotion effects

Table 5.4.3.Study 3.5 (DAR Table B.9.2.1.40) Percent inhibition of biomass integral (area under the growth curve) after 1, 2, 3 and 4 days

XDE-208 [mg/L]	1 d	2 d	3 d	4 d
0	0	0	0	0
3.13	-3.6	3.6	3.9	15.4*
6.25	19.8*	9.3*	-2.6	10.2*
12.5	11.1*	11.2*	4.7	18.9*
25	2.8	16.5*	19.6*	30.3*
50	30.3*	35.3*	37.8*	45.3*
100	32.5*	47.0*	51.8*	59.0*

* significant differences from control (Dunnett's test, $p \leq 0.05$)
Negative values mean growth promotion effects.

Effects of XDE-208 on algal growth of *Anabaena flos-aquae* are sumarized in Table 5.4.3.Study 3.6 (DAR Table B.9.2.1.41.):

Table 5.4.3.Study 3.6 (DAR Table B.9.2.1.41) Effects of XDE-208 on algal on the cyanobacteria *Anabaena flos-aquae*

EC Type	EC Value [mg a.i./L]			
	24 h	**48 h**	**72 h**	**96 h**
E_rC_{50} (growth rate)	> 100 (nominal) > 104 (mean measured)	> 100 (nominal) > 104 (mean measured)	> 100 (nominal) > 104 (mean measured)	> 100 (nominal) > 104 (mean measured)
E_yC_{50} (yield)	> 100 (nominal) > 104 (mean measured)	98 (nominal) 102 (mean measured)	87.7 (nominal) 91.2 (mean measured)	14.1 (nominal) 14.7 (mean measured)
E_bC_{50} (biomass)	> 100 (nominal) > 104 (mean measured)	> 100 (nominal) > 104 (mean measured)	94.5 (nominal) 98.3 (mean measured)	71.3 (nominal) 74.2 (mean measured)
LOEC [mg a.i./L]	6.25 (nominal) 6.50 (mean measured)	3.13 (nominal) 3.26 (mean measured)	25 (nominal) 26 (mean measured)	3.13 (nominal) 3.26 (mean measured)
NOEC [mg a.i./L]	3.13 (nominal) 3.26 (mean measured)	< 3.13 (nominal) < 3.26 (mean measured)	12.5 (nominal) 13.0 (mean measured)	< 3.13 (nominal) < 3.26 (mean measured)

Reliability of the study

The reported study is GLP compliant and conducted to a standard study protocol. Several deviations to the study plan were noted, but they are considered minor and they are not supposed to result in any significant effects on the study. The test results are in compliance with the guideline's validity criteria. The study is acceptable for regulatory use.

The test duration according to OECD guideline 201 is normally 72 hours provided that the validity criteria are fulfilled. Since all the validity criteria were met after 72 hours of the test duration, the use of the EC_{50} values for 72 hours in the risk assessment is acceptable.

Since the measured concentrations remained between 80 and 120% of the nominal concentrations SANCO/3268/2001 recommends that endpoints should normally be expressed in terms of nominal concentrations. Because the slight differences between nominal and mean measured concentrations will not fundamentally change the toxicity value, the use of toxicity endpoints based on mean measured concentrations is therefore considered to be acceptable for risk assessment purposes.

The regulatory endpoints are 72-hour E_rC_{50} >104 mg XDE-208/L, E_yC_{50} 91.2 mg XDE-208/L, E_bC_{50} 98.3 mg XDE-208/L and NOEC 13.0 mg XDE-208/L (based on mean measured concentrations).

TABLE 5.4.3.STUDY 3.7 (Sulfoxaflor DAR, Volume 3 - B.9.2.1.4.iv) Toxicity to the freshwater diatom (*Navicula pelliculosa*)

Method	**Test organism**	**Test design**	**Results (mg a.s./L)**			**Remarks**	**Reference**
			Endpoints	**NOEC [mg/L]**	**LC_{50}/EC_{50} [mg/L]**		
OECD 201, OPPTS 850.5400	Freshwater diatom (*Navicula pelliculosa*)	growth inhibition, 96h, static	biomass yield growth rate	3.7	85.7 >101 >101	mm	Dengler, D. 2009d

Citation: Dengler, D. (2009d) Sulfoxaflor: Testing of Effects of Sulfoxaflor on the Diatom *Navicula pelliculosa* in a 96 h Static Test. Eurofins-GAB GmbH, Eutinger Str. 24, D-75223 Niefern-Öschelbronn, Germany, Study code: S08-03026 Dow AgroSciences unpublished report, Study Number 080441, 07 September 2009

Guidelines: OECD Guideline 201

OPPTS 850.5400 (1996)

GLP compliance: Yes.

Test material:

Test item: Sulfoxaflor

Purity: 95.6% w/w

Description: White solid

Lot No./Batch No.: E2162-34

Material and methods:

A 96-hour static test was performed with concentrations of 0 (control), 0.41, 1.23, 3.7, 11.11, 33.33 and 100 mg/L. The controls were made in six replicates, the test item flasks were prepared in triplicate. The test flasks were inoculated with cells from exponentially growing cultures to an initial cell density of $1 \cdot 10^4$ cells/mL. By comparing the cell division under test conditions with and without the influence of test item, the inhibitory effect (EC, effect concentration) on the cell multiplication was calculated. After 1, 2, 3 and 4 days of growth, the cell numbers were measured by fluorescence detection, and the influence on growth was determined.

Statistical analysis: To estimate the LOEC, and hence the NOEC for each of the three growth indices (specific growth rate, algal yield and biomass integral), the mean value for each growth descriptor at each test concentration was compared with the corresponding control mean (all six control replicates pooled), using the multiple Dunnett's test. Before pooling the data for the control, they were statistically tested to check that none of the six replicates was significantly different from the others, using a two-tailed t-test. The Shapiro-Wilk's test was used to test the normality of the data. After confirming that the percent inhibition data were normally distributed, the EC50 values for each of the three growth indices were calculated by probit analysis.

Results

Test conditions (light intensity, temperature and pH) remained within acceptable testing limits for algae.

The concentration course of XDE-208 was verified in test medium by analysing the contents at the beginning and at the end of the test (96 h). Samples were analysed at 0.41 and 100 mg/L and control. The concentration courses can be seen in Table B.9.2.1.42. The mean measured concentration of XDE-208 was 101 % of nominal. The test item was stable during the entire test period. The toxicological evaluation, therefore, was done with nominal test concentrations and calculated mean measured concentrations determined by multiplying the overal mean recovery of 104% by the nominal concentrations.

Table 5.4.3.Study 4.1 (DAR Table B.9.2.1.42) Determined concentrations of XDE-208

Test item nominal [mg/L]	XDE-208 nominal [mg/L]	Time [d]	XDE-208		
			mg/L	% of nominal	% mean
0	0	0 96	< LOQ < LOQ	- -	-
3.13	0.39	0 96	0.41 0.388	105 99	102
100	95.60	0 96	94.09 97.23	98 102	100

- not calculated

The average cell numbers for each concentration and time of sampling are shown in Table B.9.2.1.43. The percentage inhibition of average specific growth rate, yield, and biomass integral, calculated for t = 4 d, is presented in Tables B.9.2.1.44-46. The nominal exposure concentrations were used in the result calculation. The results were checked by Dunnett's t-test and no significant differences from controls were found.

Table 5.4.3.Study 4.2 (DAR Table B.9.2.1.43) Average cell number for each sampling time and concentration

XDE-208 [mg/L]	Average cell numbers/mL *				
	0 h	24 h	48 h	72 h	96 h
0	1.00	3.04	6.68	18.37	37.15
0.41	1.00	2.77	5.74	20.73	34.60
1.23	1.00	4.15	5.68	21.05	36.29
3.7	1.00	3.04	8.56	21.22	35.84
11.11	1.00	2.21	7.35	15.38	28.65
33.33	1.00	1.59	5.62	15.64	23.95
100	1.00	0.71	3.17	8.97	19.86

* Algae counts are divided by 10000. At the start, the cell density was adjusted to $1.0 \cdot 10^4$ cells/mL

Table 5.4.3.Study 4.3 (DAR Table B.9.2.1.44) Percent inhibition of growth rate after 1, 2, 3 and 4 days

XDE-208 [mg/L]	1 d	2 d	3 d	4 d
0	0	0	0	0
0.41	8.3	8.3	-4.1	2.0
1.23	-27.9	8.8	-4.7	0.6
3.7	-0.2	-13.2	-5.0	1.0
11.11	30.1*	-5.1	6.1*	7.2*
33.33	58.5*	9.0	5.5*	12.2*
100	132.2*	40.4*	24.8*	17.4*

* significant differences from control, $p \leq 0.05$
Negative values mean growth promotion effects

Table 5.4.3.Study 4.4 (DAR Table B.9.2.1.45) Percent inhibition of yield after 1, 2, 3 and 4 days

XDE-208 [mg/L]	1 d	2 d	3 d	4 d
0	0	0	0	0
0.41	13.2	16.5	-13.6	7.1
1.23	-54.4	17.6	-15.4	2.4
3.7	0.0	-33.1	-16.4	3.6
11.11	40.7*	-11.8	17.2*	23.5*
33.33	71.1*	18.7	15.7*	36.5*
100	114.2*	61.8*	54.1*	47.8*

* significant differences from control, $p \leq 0.05$
Negative values mean growth promotion effects

Table 5.4.3.Study 4.5 (DAR Table B.9.2.1.46) Percent inhibition of biomass integral (area under the growth curve) after 1, 2, 3 and 4 days

XDE-208 [mg/L]	1 d	2 d	3 d	4 d
0	0	0	0	0
0.41	12.7	15.0	0.1	0.3
1.23	-54.9	-12.5	-8.8	-5.5
3.7	0.0	-19.3	-20.2	-9.5
11.11	40.2*	10.0	10.1	17.1*
33.33	71.6*	40.6*	23.6*	27.4*
100	113.7*	83.6*	64.2*	55.3*

* significant differences from control, $p \leq 0.05$
Negative values mean growth promotion effects.

Effects of XDE-208 on algal growth of *Navicula pelliculosa* are sumarized in Table B.9.2.1.47:

Table 5.4.3.Study 4.6 (DAR Table B.9.2.1.47) Effects of XDE-208 on the freshwater diatom *Navicula pelliculosa*

EC Type	EC Value [mg a.i./L]			
	24 h	**48 h**	**72 h**	**96 h**
E_rC_{50} (growth rate)	27.2 (nominal) 27.5 (mean measured)	> 100 (nominal) > 101 (mean measured)	> 100 (nominal) > 101 (mean measured)	> 100 (nominal) > 101 (mean measured)
E_yC_{50} (yield)	11.7 (nominal) 11.8 (mean measured)	> 100 (nominal) > 101 (mean measured)	> 100 (nominal) > 101 (mean measured)	> 100 (nominal) > 101 (mean measured)
E_bC_{50} (biomass)	11.7 (nominal) 11.8 (mean measured)	37.4 (nominal) 37.8 (mean measured)	69.1 (nominal) 69.8 (mean measured)	84.9 (nominal) 85.7 (mean measured)
LOEC [mg a.i./L]	11.11 (nominal) 11.2 (mean measured)	33.33 (nominal) 33.7 (mean measured)	11.11 (nominal) 11.2 (mean measured)	11.11 (nominal) 11.2 (mean measured)
NOEC [mg a.i./L]	3.7 (nominal) 3.7 (mean measured)	11.11 (nominal) 11.2 (mean measured)	3.7 (nominal) 3.7 (mean measured)	3.7 (nominal) 3.7 (mean measured)

Reliability of the study
The reported study is GLP compliant and conducted to a standard study protocol without significant deviations. The test results are in compliance with the guideline's validity criteria. It is acceptable for regulatory use.

Since the measured concentrations remained between 80 and 120% of the nominal concentrations SANCO/3268/2001 recommends that endpoints should normally be expressed in terms of nominal concentrations. Because the slight differences between nominal and mean measured concentrations will not fundamentally change the toxicity value, the use of toxicity endpoints based on mean measured concentrations is therefore considered to be acceptable for risk assessment purposes.

The regulatory endpoints are 96-hour E_rC_{50} > 101 mg XDE-208/L, E_yC_{50} 101 mg XDE-208/L, E_bC_{50} 85.7 mg XDE-208/L and NOEC 3.7 mg XDE-208/L (based on mean measured concentrations).

Study 5: Toxicity to the aquatic plant *Lemna gibba* (Sulfoxaflor DAR, Volume 3 - B.9.2.1.5.1)

Method	Test organism	Test design	Results (mg a.s./L)			Remarks	Reference
			Endpoints	NOEC [mg/L]	LC_{50}/EC_{50} [mg/L]		
OECD 221, OPPTS 850.4400	Duckweed (*Lemna gibba*)	growth inhibition, 7d, semi-static	biomass frond yield growth rate	100 100 100	>100 >100 >100	nom	Kuhl, R, Wydra, V. 2009b

Citation: Kuhl, R, Wydra, V. (2009b): Sulfoxaflor technical: Toxicity of Sulfoxaflor technical to the Aquatic Plant *Lemna gibba* in a Semi-Static Growth Inhibition Test. IBACON GmbH, Arheiliger Weg 17, 64380 Rossdorf, Germany, Laboratory Project Number: 46841240. Dow AgroSciences unpublished report, Study Number 080443. 20 July 2009

Guidelines: OECD Guideline 221

OPPTS 850.4400

GLP compliance: Yes.

Test material:

Test item: Sulfoxaflor

Purity: 95.6% w/w

Description: White solid

Lot No./Batch No.: E2162-34

Material and methods:

In a 7-days semi-static test, 12 fronds of *Lemna gibba* were exposed to the test item Sulfoxaflor technical at the nominal concentrations of 100, 50, 25, 12.5, 6.25, 3.14 and 1.56 mg a.s./L. The test medium of the highest test concentration of nominal 100 mg/L was prepared by dissolving approximately 150 mg test item into 1500 mL culture medium by intense stirring and short ultrasonic treatment. Adequate volumes of this test medium were diluted with culture medium to prepare the test media. The test media were freshly prepared just before introduction of the aquatic plants (= start of the test and each test medium renewal). In the control, test medium was used without addition of the test item. The test media were renewed at day 3 and 5.

Three replicates per test treatment were used, resulting in 36 fronds of *Lemna gibba* per test

treatment. The frond number was counted on days 3, 5 and 7. At the end of the test, the dry weight was measured.

The pH and temperature were measured in the freshly prepared and aged test media of each treatment daily.

Statistical analysis: The EC_{50}-values could not be determined due to absence of toxicity of the test item. For the determination of the LOEC and NOEC values significant differences at the test concentrations compared to the control values were tested by the Dunnett's test. For the parameter yield (frond number), the evaluation showed a significant increase in growth for the 6.25 mg test item/L treatment. However, since in the concentrations above and below, no significant reduction or increase could be observed, this inhibition is not considered a toxicological effect but biological variance. The software used to perform the statistical analysis was ToxRat Professional.

Results

Test conditions (light intensity, temperature and pH) remained within acceptable testing limits for algae.

Analytical samples were collected from all freshly prepared and aged test media and analyzed using a high performance liquid chromatography (HPLC-method). The concentrations of the test item XDE-208 technical were measured in the duplicate test medium samples from all test concentrations at days 0, 3, 5 (freshly prepared test media respectively) and at days 3, 5, 7 (aged test media, respectively). From the control samples only one of the duplicate samples was analysed from these samplings. A summary of analytical results is given in Table B.9.2.1.48. In the freshly prepared test media 99 % of the nominal test concentrations were found. In the aged test media 97 % of the nominal values were determined. Thus, during the test period of 48 and 72 hours the *Lemna* were exposed to a mean of 98 % of nominal. Therefore, all reported results are related to nominal concentrations of the test item.

Table 5.4.3.Study 5.1 (DAR Table B.9.2.1.48) Summary of analytical results

sample description [µg test item/L]	% of nominal[1]	RSD [%]	n
control	n.a.	n.a.	6
1.56	97	6	12
3.14	101	7	12
6.25	98	8	12
12.5	97	7	11
25.0	98	6	12
50.0	98	6	12
100	99	10	12

[1] mean value of all measured samples per treatment group
RSD relative standard deviation per treatment group
n.a. not applicable
n number of samples used for calculation of mean values

XDE-208 exhibited no treatment-related effect on growth rate and yield of frond numbers and dry weight, frond shape and color of *Lemna gibba*. The statistical endpoints are summarized in Table B.9.2.1.49.

Table 5.4.3.Study 5.1 (DAR Table B.9.2.1.49) Effects of XDE-208 in *Lemna gibba*: Statistical endpoints

Endpoint	Frond Yield	Frond Average Specific Growth Rate	Biomass Yield as Dry Weight	Biomass Average Specific Growth Rate as Dry Weight
NOEC	≥ 100	≥ 100	≥ 100	≥ 100
LOEC	> 100	> 100	> 100	> 100
EC_{50}	> 100	> 100	> 100	> 100

Validity criteria: Doubling time of frond number in control must be less than 2.5 days (60 h). Actual time was 1.8 days, validity criterion was met.

Reliability of the study
The reported study is GLP compliant and conducted to a standard study protocol without significant deviations. The test results are in compliance with the guideline's validity criteria. It is acceptable for regulatory use.

Since the measured concentrations remained between 80 and 120% of the nominal concentrations SANCO/3268/2001 recommends that endpoints should normally be expressed in terms of nominal concentrations.

Based on nominal concentrations, the regulatory endpoints are a 7-day E_rC_{50} and E_yC_{50} > 100 mg XDE-208/L, and 7-day NOEC ≥ 100 mg XDE-208/L, the highest concentration tested.

5.4.4 Other aquatic organisms (including sediment)

Study 1: Acute toxicity to marine or estuarine invertabrate – Eastern oyster (*Crassostrea virginica*) (Sulfoxaflor DAR, Volume 3 - B.9.2.1.3.v)

Method	Test organism	Test design	Results (mg a.s./L)			Remarks	Reference
			Endpoints	NOEC [mg/L]	LC_{50}/EC_{50} [mg/L]		
OPPTS 850.1025, EPA 72-3	Eastern oyster (*Crassostrea virginica*)	acute, 96h flow-through	shell growth	67.3	86.5	mm	Hicks S.L. 2008c

Citation: Hicks S.L. (2008c): Sulfoxaflor: Effect on New Shell Growth of the Eastern Oyster (*Crassostrea virginica*). ABC Laboratories, Inc., 7200 E. ABC Lane, Columbia MO 65202, ABC Study Number 63667. Dow AgroSciences unpublished report, Study Number 080070. November 14, 2008.

Guidelines: OPPTS 850.1025

FIFRA 72-3

GLP compliance: Yes.

Test material:

Test item: Sulfoxaflor

Purity: 95.6% w/w

Description: White solid

Lot No./Batch No.: E2162-34

Material and methods:

A 96-hour flow-through toxicity test was performed with test concentrations of 0 (control), 26, 43, 72, 120, and 200 mg Sulfoxaflor/L. Diluter stock solutions were prepared on August 12, 13, 14, 15, 16, 17, and 18, 2008, at a target concentration of 200 mg a.s./L by diluting approximately 41.8410 g (40.000 g corrected for purity) of Sulfoxaflor with 200 L of dilution water. Each diluter stock solution was prepared in the following manner: 1) The test substance sample was transferred to a one-gallon glass jar with salt water while stirring and sonicating, and additional saltwater was added until the jar was nearly full; 2) The contents of the jar were transferred to a barrel and the solution volume in the barrel was brought to a volume of 200 L; 3) The barrel contents were mixed with an overhead stirrer for at least 30 minutes. The diluter stock solution was used as the highest test substance treatment solution and the four lower treatment solutions were prepared using appropriate volumes of the diluter stock solution at each cycle of the proportional diluter system.

A 2,000-mL proportional diluter system similar to that described by Mount and Brungs, with a utility pump, was used for the intermittent introduction of control and Sulfoxaflor test solutions into each test chamber. The test chambers were arranged in a temperature-controlled water bath using a computer-generated random number table to assign specific treatment location. The diluter system delivered approximately 1,000 mL of each solution to the appropriate test chambers with each cycle during the test.

Operation of the diluter system and delivery of the test substance was initiated on August 12, 2008. At 8:05 am on August 15, the diluter was observed to not be cycling. Cycle counts indicated that the only 2 cycles had occurred after 10:15 pm the previous evening. Diluter function/cycling was restored at 8:15 am on August 15 and the diluter was allowed to cycle 9 times before it was temporarily stopped for analytical sampling.

A total of 120 actively growing oysters were impartially selected from the oyster culture and the shell margins were cleared of new shell growth at test initiation. As in the culture tanks, they were placed with the cupped valve down and the open end of the valves oriented into the flow of the recirculating water. A marine micro algal concentrate (Instant Algae Shellfish Diet 1800, Reed Mariculture, Inc.) was added manually (i.e., 3 mL added three times each day during exposure with exceptions of test initiation and termination, when 3 mL was added only once) to each test chamber during the exposure. Observations for mortality and other signs of test substance effect (e.g., slow valve closure and lack of feeding activity as evident from lack of faecal deposits) were made daily (±1 hour from test initiation). New shell growth at test termination was measured to the nearest 0.1 mm with a vernier caliper [Manostat (15-100-100) Mecanic Type 6911].

Test solution salinity, temperature, pH, and dissolved oxygen concentration were measured daily in each test chamber. A continuous temperature recording from the control test chamber was maintained for the duration of the test using an electronic data logger.

Statistical Analysis: All statistical analyses were performed using SAS software. A one-tailed Dunnett's test was conducted at the 0.05 level of significance, with comparison to the control group. The alternate hypothesis was that the mean new shell growth for the treatment group had been

reduced in comparison to the control mean new shell growth. Prior to the Dunnett's test, a Shapiro-Wilk test for normality and Levene's test for homogeneity of variance over treatments were conducted. The assumptions of normality and homogeneity of variance were met for the raw data values; therefore, a parametric analysis was performed on the raw data. The EC50 and 95% confidence limits for new shell growth data were calculated by a four-parameter logistic (sigmoid-shaped) model, two parameters fixed (100 and 0% inhibition), fit to the data with percent inhibition as the dependent variable and log concentration as the independent variable.

Deviations to the study protocol:

The flow rate to each test chamber was not at least 1 L/hour per oyster between 10:15 pm on August 14 (i.e. day 0 of the exposure) and 8:05 am on August 15 (i.e., day 1 of the exposure). The diluter system malfunctioned and had only cycled twice during this time; therefore, fresh test solutions were not being introduced to each test chamber at a rate to achieve 1 L/hour per oyster.

Stability data generated as part of an earlier study (i.e. ABC Study No. 63666; DAS No. 080069) indicated Sulfoxaflor was stable in laboratory saltwater for a period of at least 96 hours. Based on these results, the interruption in test solution addition to the test chambers between 0 and 24 hours of the exposure did not affect the exposure concentration during this period. Additionally, new shell growth by the control oysters and control oysters survival did meet the acceptability criteria; therefore, the deviation did not affect the study integrity or interpretation of the test results.

Results
Salinity, temperature, and pH remained within acceptable limits throughout the 96-hour definitive test. The control and test solutions were clear and colourless with no visible signs of undissolved test substance, precipitate, or surface film throughout the study.

Analytical confirmation of the test substance, XDE-208, in test solutions was performed at –N (day prior to initiation), 0, and 96 hours, as well as day 1 of the definitive test. Measured concentrations of XDE-208 in test substance treatments prior to test initiation (day –N) were 26.4, 44.6, 78.5, 125, and 205 mg a.i./L, which represented recoveries of 102 to 109% of the nominal test substance treatment concentrations. Measured concentrations of XDE-208 in test substance treatments at 0 hour were 24.2, 39.4, 68.5, 108, and 181 mg a.i./L, which represented recoveries of 90 to 95% of the nominal test substance treatment concentrations. Measured concentrations at approximately 24 hours were 25.2, 40.4, 63.5, 103, and 166 mg a.i./L, which represented recoveries of 83 to 97% of the nominal test substance treatment concentrations. Measured concentrations at 96 hours were 24.3, 43.0, 70.0, 124, and 187 mg a.i./L, which represented recoveries of 93 to 103% of the nominal test substance treatment concentrations. Mean measured concentrations (i.e., mean of the 0, 24, and 96 hour measured concentrations) were 24.6, 40.9, 67.3, 112, and 178 mg a.i./L, which represented recoveries of 89 to 95% of the nominal test substance treatment concentrations. No residues of XDE-208 were detected in the control solution above the MQL of 2.50 mg a.i./L. Since the measured concentrations approximated the nominal concentrations (i.e., within 80 to 120% of nominal) and were stable, the biological response results were based upon the nominal concentrations and the mean measured concentrations.

After 96 hours of exposure, there was no mortality in the control or any of the test substance treatments. There was a noticeable reduction in fecal material observed at 48, 72, and 96 hours in the 120 and 200 mg a.i./L nominal test substance treatments. Mean new shell growth values were 2.9, 3.2, 2.2, 2.2, 0.81, and 0.53 mm in the control, 26, 43, 72, 120, and 200 mg a.i./L nominal treatments, respectively (B.9.2.1.13). The percent difference in new shell growth ranged from -82% in the 200 mg a.i./L nominal treatment to +10% in the 26 mg a.i./L nominal treatment, as compared

to the control new shell growth (i.e., 2.9 mm).

Table 5.4.4.Study 1.1 (DAR Table B.9.2.1.13) Effect of XDE-208 on new shell growth in eastern oyster

Treatment (mg a.i./L)		Observation Period: 96-hr	
Nominal	**Mean Measured**	**Mean Length ± SD (mm)**	**% Change from Control**
Negative Control	<MQL	2.9 ± 0.56 (range: 2.0 to 4.5)	NA
26	24.6	3.2 ± 0.64 (range: 2.0 to 4.8)	+10
43	40.9	2.2 ± 0.68 (range: 1.1 to 3.5)	-24
72	67.3	2.2 ± 0.64 (range: 1.0 to 3.5)	-24
120	112	0.81 ± 0.73 (range: 0 to 2.3)	-72*
200	178	0.53 ± 0.53 (range: 0 to 1.6)	-82*
NOEC		72 mg a.i./L (nominal) 67.3 mg a.i./L (mean measured)	
EC50		93 mg a.i./L (nominal) 86.5 mg a.i./L (mean measured)	
* Significantly different from control, Dunnett's test p<0.05.			

Reliability of the study
The reported study is GLP compliant and conducted to a standard study protocol without significant deviations. The test results are in compliance with the guideline's validity criteria. It is acceptable for regulatory use.

Since the measured concentrations remained between 80 and 120% of the nominal concentrations SANCO/3268/2001 recommends that endpoints should normally be expressed in terms of nominal concentrations. Because the slight differences between nominal and mean measured concentrations will not fundamentally change the toxicity value, the use of toxicity endpoints based on mean measured concentrations is therefore considered to be acceptable for risk assessment purposes.

Based on mean measured concentrations, the regulatory endpoint is a 96-hour EC_{50} 86.5 mg XDE-208/L.

Study 2: Acute toxicity to the sediment dwelling invertebrate Chironomus dilutus (Sulfoxaflor DAR, Volume 3 - B.9.2.1.3.vi)

Method	**Test organism**	**Test design**	**Results (mg a.s./L)**			**Remarks**	**Reference**
			Endpoints	**NOEC [mg/L]**	**LC_{50}/EC_{50} [mg/L]**		
OECD 202, OPPTS 850.1010	Chironomus dilutus	acute, 96h, spiked water, static	mortality subleth. effects	<0.131	0.622	mm	Gerke, A. 2008d

Citation: Gerke, A. 2008d: Sulfoxaflor: Acute 96 Hour Toxicity to the Midge, *Chironomus dilutus*, determined Under Static Test Conditions. ABC Laboratories, Columbia, Missouri, ABC 63967. Dow AgroSciences unpublished report, Study Number 080362. December 1, 2008.

Guidelines: OECD Guideline 202

OPPTS 850.1010

JMAFF 2-7-2-1

GLP compliance: Yes.

Test material:

Test item: Sulfoxaflor

Purity: 95.6% w/w

Description: White solid

Lot No./Batch No.: E2162-34

Material and methods:

A 96-hour static test was performed with test concentrations of 0 (control), 0.13, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16, and 32 mg a.s./L. All solution preparations were corrected for the purity of the test substance. A 0.20 mg Sulfoxaflor/mL primary stock solution was prepared at test initiation by suspending 0.1050 g Sulfoxaflor (0.1004 g a.s.) into a 500 mL volume of dilution water. Aliquots of the primary stock solution were used to prepare each test concentration. The control consisted of dilution water only.

Five midge larvae were impartially added to a set of labelled containers with each container representing one treatment replicate. Each container was then randomly assigned to a treatment replicate by random number generator. The individuals within each container were then released from the container into the corresponding test chamber. There were four replicates per treatment level, resulting in 20 midge per test treatment. The test chambers were grouped by treatment in a water bath. No aeration was provided to any test chamber during the test. Observations for mortality and sublethal responses were made every 24 hours (±1 hour) for the duration of the test.

Temperature, pH, and dissolved oxygen concentration were measured in each test chamber on a daily basis. Alkalinity, hardness, and conductivity were measured in a sample of the dilution water at test initiation. A small amount of fine silica sand was added to each vessel to allow a minimal substrate for the larvae to adhere to.

Statistical analysis: Estimates of LC50 values and their 95% confidence limits were calculated using the probit method and Trimmed Spearman-Karber method. When the P value for Goodness of Fit was >0.05 and there was no other evidence of questionable convergence, the probit method was selected for reporting. When this criterion was not achieved, the Trimmed Spearman-Karber method was selected for reporting.

Results

Water quality parameters (pH, temperature and dissolved oxygen) remained within acceptable testing limits. The control and test solutions were clear and colourless with no visible signs of undissolved test substance, precipitate, or surface film throughout the study.

XDE-208 concentrations within the test solutions were measured at 0, 48 and 96 hours. Measured concentrations of XDE-208 in the test solutions at test initiation were <MQL (control), 0.123, 0.210, 0.442, 0.880, 1.87, 3.78, 7.25, 14.8, and 29.1 mg XDE-208/L, which represented recoveries of 84 to 95% of the nominal treatment concentrations. The measured concentrations in the 48-hour test solutions were <MQL (control), 0.125, 0.214, 0.438, 0.915, 1.83, 4.01, 7.45, 15.0, and 28.4 mg XDE-208/L, which represented recoveries of 86 to 100% of the nominal treatment concentrations. The measured concentrations in the 96-hour test solutions were <MQL (control), 0.146, 0.222, 0.468, 1.09, 1.91, 4.66, 8.20, 15.6, and 33.2 mg XDE-208/L, which represented recoveries of 89 to 117% of the nominal treatment concentrations. The mean measured concentrations in the test solutions during the 96-hour study were <MQL (control), 0.131, 0.215, 0.449, 0.962, 1.87, 4.15, 7.63, 15.1, and 30.2 mg XDE-208/L, which represented recoveries of 86 to 104% of the nominal treatment concentrations.

After 96 hours of exposure, mortality was 10, 35, 40, 55, 70, 65, 75, 80, 70, and 65% in the 0 (control), 0.13, 0.25, 0.50, 1.0, 2.0, 4.0, 8.0, 16, and 32 mg XDE-208/L treatments (Table B.9.2.1.14). Sublethal effects (organisms that were lethargic or displaying erratic movement) were noted in all test treatments during the definitive test (Table B.9.2.1.15).

Table 5.4.4.Study 2.1 (DAR Table B.9.2.1.14) Effect of XDE-208 on mortality of *Chironomus dilutus*

Treatment (mg a.i./L)		**Cumulative mortality**				
Nominal	**Mean Measured**	**24-hr**	**48-hr**	**72-hr**	**96-hr**	**Total (%)**
Negative control	<MQL	2	2	2	2	10
0.13	0.131	0	0	6	7	35
0.25	0.215	0	1	6	8	40
0.50	0.449	1	1	8	11	55
1.0	0.962	2	6	10	14	70
2.0	1.87	0	4	10	13	65
4.0	4.15	2	6	10	15	75
8.0	7.63	2	6	11	16	80
16	15.1	0	3	6	14	70
32	30.2	0	3	10	13	65
MQL=0.050 mg a.i./L						
48 hour NOEC		<0.13 mg a.i./L (nominal) or <0.131 mg a.i./L (mean measured)				
48 hour LC50		>32 mg a.i./L (nominal) or >30.2 mg a.i./L (mean measured)				
96 hour NOEC		<0.13 mg a.i./L (nominal) or <0.131 mg a.i./L (mean measured)				
96 hour LC50		0.656 mg a.i./L (nominal) or 0.622 mg a.i./L (mean measured)				

Table 5.4.4.Study 2.2 (DAR Table B.9.2.1.15) Sub-lethal effects of XDE-208 in *Chironomus dilutus*

Treatment (mg a.i./L)		**Observation period**			
Nominal	**Mean Measured**	**No. Sublethal Effects Observed (% affected)**			
		24-hr	**48-hr**	**72-hr**	**96-hr**

Negative control	<MQL	0 (0)	0 (0)	0 (0)	0 (0)
0.13	0.131	2 (10)	20 (100)	14 (100)	13 (100)
0.25	0.215	9 (45)	19 (100)	14 (100)	12 (100)
0.50	0.449	19 (100)	19 (100)	12 (100)	9 (100)
1.0	0.962	18 (100)	14 (100)	10 (100)	6 (100)
2.0	1.87	20 (100)	16 (100)	10 (100)	7 (100)
4.0	4.15	18 (100)	15 (100)	10 (100)	5 (100)
8.0	7.63	18 (100)	14 (100)	10 (100)	4 (100)
16	15.1	20 (100)	17 (100)	14 (100)	6 (100)
32	30.2	20 (100)	17 (100)	10 (100)	7 (100)
Sublethal effects consisted of lethargic appearance and/or displaying erratic movements.					

Reliability of the study
The reported study is GLP compliant and conducted to a standard study protocol without significant deviations. The test results are in compliance with the guideline's validity criteria. It is acceptable for regulatory use.

Since the measured concentrations remained between 80 and 120% of the nominal concentrations SANCO/3268/2001 recommends that endpoints should normally be expressed in terms of nominal concentrations. Because the slight differences between nominal and mean measured concentrations will not fundamentally change the toxicity value, the use of toxicity endpoints based on mean measured concentrations is therefore considered to be acceptable for risk assessment purposes.

Based on mean measured concentrations, the regulatory endpoint is a 96-hour LC_{50} 0.622 mg XDE-208/L.

Study 3: Acute toxicity to the sediment dwelling invertebrate - whole sediment 10 day test with Chironomus dilutus (Sulfoxaflor DAR, Volume 3 - B.9.2.1.3.vii)

Method	**Test organism**	**Test design**	**Results (mg a.s./L)**			**Remarks**	**Reference**
			Endpoints	**NOEC [mg/L]**	**LC_{50}/EC_{50} [mg/L]**		
OPPTS 850.1735	Chironomus dilutus	acute, 10d, spiked sediment, static	mortality weight	0.036	0.119	mm	Gerke, A. 2008f

Citation: Gerke, A. 2008f: Sulfoxaflor: Whole Sediment 10 Day Acute Toxicity Test with Midge Larvae (*Chironomus dilutus*). ABC Laboratories, Columbia, Missouri, ABC 63673. Dow AgroSciences unpublished report, Study Number 080076. 30 September 2008.

Guidelines: OPPTS 850.1735

GLP compliance: Yes.

Test material:

Test item: Sulfoxaflor and ^{14}C-Sulfoxaflor (X11859293)

Purity: Sulfoxaflor: 95.6% w/w

^{14}C-Sulfoxaflor: 99.7% w/w, 45.2 mCi/mmol

Description: White solid

Lot No./Batch No.: Sulfoxaflor: TSN003725-0001, E2162-34

^{14}C-Sulfoxaflor: INV027474-0001, XS9-37562-34

Material and methods:

A 10-day static test was performed with nominal test concentrations of 0 (control), 0.065, 0.13, 0.25, 0.50, and 1.0 mg a.s./kg dry sediment. A 0.074 mg/mL primary application stock solution was prepared by weighing a total of 1.47 mg of ^{14}C-labeled Sulfoxaflor and 5.931 mg of non-radio labelled Sulfoxaflor into a 100-mL glass volumetric flask and bringing the flask to volume with dilution water. A dilution of the primary stock solution was utilized to prepare the stock solutions. All of the stock solutions were prepared in dilution water. To prepare the dosed sediments, a 64 mL volume of dilution water or the appropriate dosing solution was added to 200 g of dried sediment in a stainless steel pan. The solutions were mixed by hand into the dried sediment thoroughly. Then 2,339 g of wet sediment (oven dry equivalent of 1,706 g for a total dry weight of 1.896 kg) was added to each stainless steel pan and mixed thoroughly by hand. The nominal Sulfoxaflor concentrations of these dosed sediments, based on the sediment weight and stock solution concentrations were 0 (control), 0.065, 0.13, 0.25, 0.50, and 1.0 mg/kg dry sediment. Approximately 296 g (equivalent to 175 mL) of prepared sediment was added to each replicate test chamber. A 700-mL volume of dilution water was carefully added to the test chambers. A plastic deflector was placed just above the sediment surface while the water was added to minimize the disturbance of the prepared sediments.

Ten midge larvae were impartially added to a set of labelled containers with each container representing one treatment replicate. Each container was then randomly assigned to a treatment replicate by random number generator. The individuals within each container were then released from the container into the corresponding test chamber. There were four biological replicates per treatment level, resulting in 40 midge per test treatment. Aeration was provided to each test chamber through a glass pipette set at a depth of 2-3 cm above the sediment and maintained at a rate of 60 to 100 bubbles per minute. Observations for sediment activity, aeration, and water level were made daily for the duration of the test. At test termination, the entire contents of each test chamber were poured through a stainless steel mesh and the live and dead organisms were enumerated. Observations of general health and behaviour of the organisms were also noted. Any midge not accounted for on day 10 (i.e., not found) were considered dead.

Temperature, pH, and dissolved oxygen concentration were measured in each biological replicate on a daily basis. On days 0 and 10, equal volumes of sample were removed with a pipette from 1 to 2 cm above the sediment surface from each replicate and the replicates were composited by treatment for analysis of specific conductivity, total alkalinity, total hardness, and ammonia concentrations.

Statistical analysis: All statistical analyses were performed using SAS software. The program calculated the LC50 value statistic and its 95% confidence limits using the probit method and Trimmed Spearman-Karber method. When the *p* value for Goodness of Fit was >0.05 and there was no other evidence of questionable convergence, the probit method was selected for reporting. When this criterion was not achieved, the Trimmed Spearman-Karber method was selected for reporting. The no-observable-effect concentration (NOEC) and lowest-observable-effect concentration (LOEC) was determined by using Fisher's exact test. A Hochberg adjustment was used to control the experiment wise error rate for the Fisher's test at the same alpha level. The slope of the

concentration-response line was calculated by regression analysis of the transformed percent mortality values (i.e. probit values) versus the log of the test concentration.

Growth data (as dry weight) was analyzed using a one-way analysis of variance (ANOVA). Aone-tailed Dunnett's test was conducted at the $p \leq 0.05$ level of significance, with comparison to the control group. Prior to the Dunnett's test, a Shapiro-Wilk's test and a Levene's test for normality and homogeneity of variance over treatments at each day were conducted. Where the *p* values from the Shapiro-Wilk's and Levene's test were greater than 0.01, indicating normality and insignificant heterogeneity, the analysis was performed on the raw value. Where the *p* value was less than 0.01, a log transformation was used.

Deviations to the study plan:

1. Temperature from the continuous temperature recorded from the waterbath indicated that for approximately 10 hours, the temperature was not maintained at 23 ± 1ºC during the definitive study.
   Reason: During the first day of the study, the waterbath temperature ranged from 22.1 to 24.7 ºC. An adjustment was made, and temperature was maintained through the remainder of the study.

   Impact on study: None. The minor temperature deviation did not adversely affect midge survival.

2. Ashed dry weights will not be determined or reported for the surviving larvae.
   Reason: Sample integrity was compromised when the container holding the samples was broken during the processing of the ashed samples.

   Impact on study: None. Comparisons of the pre-ashed dry weights will be made against the control, which meets the requirements set by OPPTS guideline 850.1735.

3. Four biological replicates per treatment level were prepared for the definitive study.
   Reason: Technical staff prepared a total of eight replicates per treatment instead of eight biological replicates and an additional four replicates for analytical verification.

   Impact on study: None. OPPTS guideline recommends eight replicates. The minimal variability between replicates of the same treatment level suggests this deviation does not adversely affect the study integrity or the interpretation of the results.

4. Upon test termination, one replicate contained 11 midge larvae.
   Reason: An extra midge was inadvertently added to a test chamber.

   Impact on study: None. This deviation does not adversely affect the study integrity or the interpretation of the results.

Results

The daily water quality measurements from the overlying water within the test chambers remained within acceptable limits throughout the test with the exception of four dissolved oxygen values. The dissolved oxygen concentrations in the overlying water at test initiation ranged from 6.7 to 8.6 mg/L (82 to 105% saturation). The dissolved oxygen values for the remainder of the exposure ranged from 1.5 to 8.1 mg/L (18 to 99% saturation). There were 4 dissolved oxygen values that were below 40% saturation (3.28 mg/L) during the 10-day exposure that ranged from 1.5 to 3.0 mg/L (18-60% saturation). These values were recorded on days 2 and 8 of the exposure and were unlikely to have affected the interpretation of the biological results. The pH of the overlying water ranged from 7.6 to 8.5 during the study. The temperatures recorded within the test chambers ranged

from 22.5 to 23.0°C during the study.

Overlying water, interstitial (pore) water, and sediment were analyzed for total radioactive residues (TRR) by liquid scintillation counting (LSC) of duplicate samples. Duplicate samples were collected from each control or test substance treatment at test initiation and test termination. Two aliquots from each duplicate sample were analyzed for both the overlying and interstitial water samples. Three aliquots from each duplicate sample were analyzed for sediment samples. Overlying water was also analyzed for XDE-208 and the metabolite X11719474 using a high performance liquid chromatographic/mass spectrometry (HPLC/MS/MS) system. Analytical results are given in Tables B.9.2.1.16-19. All biological response evaluations were calculated based on mean measured ^{14}C-labeled XDE-208 concentrations in sediment. The sediment TRR concentrations were corrected for dry weight of sediment.

Table 5.4.4.Study 3.1 (DAR Table B.9.2.1.16) Analytical results from analysis of overlying water samples for XDE-208 total radioactive residues (TRR).

Nominal Sediment Concentration (mg/kg dry sediment)	Mean Measured Concentrations (mg TRR/L)		
	Day 0	Day 10	Mean
0 (control)	<MQL	<MQL	<MQL
0.065	0.00345	0.00915	0.00630
0.13	0.00663	0.0186	0.0126
0.25	0.0122	0.0339	0.0231
0.50	0.0236	0.0702	0.0469
1.0	0.0517	0.134	0.0929
MQL:	Day 0: 0.0000476 mg TRR/L; Day 10: 0.0000514 mg TRR/L		

Table 5.4.4.Study 3.2 (DAR Table B.9.2.1.17) Analytical results from analysis of pore water samples for XDE-208 total radioactive residues (TRR)

Nominal Sediment Concentration (mg/kg dry sediment)	Mean Measured Concentrations (mg TRR/L)		
	Day 0	Day 10	Mean
0 (control)	<MQL	<MQL	<MQL
0.065	0.0665	0.0307	0.0486
0.13	0.136	0.0626	0.0993
0.25	0.245	0.111	0.178
0.50	0.500	0.248	0.374
1.0	1.06	0.471	0.766
MQL:	Day 0: 0.0000523 mg TRR/L; Day 10: 0.0000565 mg TRR/L		

Table 5.4.4.Study 3.3 (DAR Table B.9.2.1.18) Analytical results from analysis of sediment samples for XDE-208 total radioactive residues (TRR)

Nominal Sediment Concentration (mg/kg dry sediment)	Mean Measured Concentrations (mg TRR/kg)				Other parameters
	Day -1	Day 0	Day 10	Mean (Days 0-10)	
0 (control)	<MQL	<MQL	<MQL	<MQL	Type: Natural
0.065	0.0560	0.0340	0.0151	0.0246	Total organic carbon (%): 0.6
0.13	0.113	0.0640	0.0336	0.0488	Total organic matter (%): 1.1
0.25	0.218	0.116	0.0623	0.0892	Clay (%): 12
0.50	0.424	0.219	0.129	0.174	Sand (%): 68
1.0	1.03	0.467	0.249	0.358	Silt (%): 20
MQL:	Day -1: 0.00126 mg TRR/kg; Day 0: 0.000760 mg TRR/kg; Day 10: 0.000781 mg TRR/kg				

Table 5.4.4.Study 3.4 (DAR Table B.9.2.1.19) Analytical results from analysis of overlying water samples for XDE-208 and metabolite X11719474 by confirmatory LC/MS/MS.

Nominal Sediment Concentration of XDE-208 (mg/kg dry sediment)	Mean Measured Concentrations (mg/L)		
	Day 0 Mean	Day 0 Mean	Day 0-10 Mean
XDE-208			
0 (control)	<MQL	<MQL	<MQL
1.0	0.0751	0.154	0.115
X11719474			
0 (control)	<MQL	<MQL	<MQL
1.0	<MQL	0.0368	0.0201[a]
MQL for XDE-208: Day 0 and Day 10: 0.00667 mg a.i./L MQL for X11719474: Day 0 and Day 10: 0.00667 mg a.i./L			
[a] Mean calculation used ½ of the MQL for the Day 0 value.			

After 10 days of exposure, the effects of XDE-208 on survival and weight of *Chironomus dilutus* exposed via whole sediment were assessed. The results are summarized in Tables B.9.2.1.20-21.

Table 5.4.4.Study 3.5 (DAR Table B.9.2.1.20) Effect of XDE-208 on survival of *Chironomus dilutus* exposed via whole sediment

Mean Measured Sediment Concentration (mg TRR/kg dry sediment)	Day 10 Survival	
	Number Surviving/Number Tested	Percent Survival (%)
<MQL (Control)	38/40	95
0.0246	40/40	100
0.0488	37/40	93
0.0892	39/41	95
0.174	17/40	43*
0.358	0/40	0*
* Significantly different from control, Fisher's exact test p<0.05.		
NOEC	0.0892 mg TRR/kg dry sediment	
LOEC	0.174 mg TRR/kg dry sediment	
LC50	0.161 mg TRR/kg dry sediment (95% CL: 0.142 to 0.182 mg TRR/kg dry sediment)	
MQL:	Day -1: 0.00126 mg TRR/kg; Day 0: 0.000760 mg TRR/kg; Day 10: 0.000781 mg TRR/kg	

Table 5.4.4.Study 3.6 (DAR Table B.9.2.1.21) Effect of XDE-208 on weight of *Chironomus dilutus* exposed via whole sediment

Mean Measured Sediment Concentration (mg TRR/kg dry sediment)	Day 10 Dry Weight (mg)	
	Replicate Means	Overall Mean
<MQL (Control)	2.75, 2.70, 2.66, 2.65	2.69
0.0246	2.44, 2.21, 2.53, 2.84	2.51
0.0488	2.39, 2.14, 3.10, 2.05	2.42
0.0892	1.90, 1.87, 1.74, 1.87	1.85*
0.174	0.79, 1.26, 1.23, 0.77	1.01*
0.358	--	--
* Significantly different from control, Dunnett's test p<0.05.		
NOEC	0.0488 mg TRR/kg dry sediment	
LOEC	0.0892 mg TRR/kg dry sediment	
EC50	Not calculated	
MQL:	Day -1: 0.00126 mg TRR/kg; Day 0: 0.000760 mg TRR/kg; Day 10: 0.000781 mg TRR/kg	

Reliability of the study
The reported study is GLP compliant and conducted to a standard study protocol. Several deviations to the study plan were noted, but they are considered minor and they are not supposed to result in any significant effects on the study. The test results are in compliance with the guideline's validity criteria. The study is acceptable for regulatory use.

Since the test substance, XDE-208, was metabolized to some extent in the whole sediment test system over the 10 days of the study to form the metabolite X11719474, as determined by measurement of the metabolite in the overlying water, the suggested correction of the toxicity endpoints are as follows: Comparing the overlying water concentrations of X11719474 (0.0368 mg a.i./L) to XDE-208 (0.134 mg TRR/L) at the 10 day sampling time point suggests that a proportion of the mass of TRR could be explained by formation of residues of the metabolite X11719474. It has been shown (as summarized previously) that X11719474 exhibits very low toxicity to *Chironomus*, consistent with its lack of insecticidal activity. Therefore, expression of the 10 day LC_{50} and NOEC in terms of Total Radioactive Residues (TRR) of XDE-208 may slightly over-estimate the actual endpoints if they were expressed as residues of XDE-208 alone. To properly account for the approximate 28% loss (on a mass basis) of XDE-208 from the test system during the study, it is appropriate to correct the TRR-based endpoints. To account for the ratio of molecular weights, 1 mole of XDE-208 with a molecular weight of 277 g/mol yields 1 mole of X11719474 with a molecular weight of 295 g/mol and thus a molar ratio of XDE-208/X11719474 of 0.938, the proportion of XDE-208 TRR accounted for by residues of X11719474 must be also corrected by this difference in molecular weights. In other words, 1 mg of X11719474 is derived from 0.938 mg of XDE-208, and referring this relationship to the measured concentration of X11719474 in the overlying water gives 0.0368 mg X11719474/L x 0.938 mg XDE-208./mg X11719474 = 0.0345 mg a.i./L converted by metabolism. As a percentage of the Total Radioactive Residues of XDE-208 in the overlying water, it is estimated that 26% (=0.0345 mg/L / 0.134 mg TRR/L x 100%) of the TRR was converted by metabolism during the study and so the TRR represents 100%-26%= 74% as XDE-208. Thus, the corrected 10 day endpoints for *Chironomus dilutus* are calculated as follows:
Corrected 10 day LC_{50} = 0.161 mg TRR/kg x 0.74 = 0.119 mg XDE-208/kg sediment, and;
Corrected 10 day NOEC = 0.0488 mg TRR/kg sediment x 0.74 = 0.036 mg XDE-208/kg sediment.

The correction of the toxicity endpoints suggested was accepted..

Based on mean measured ^{14}C-labeled XDE-208 concentrations in sediment, a 10-day LC_{50} is 0.161 mg XDE-208 TRR/kg, corrected 10-day LC_{50} is 0.119 mg XDE-208/kg sediment. Based on mean measured ^{14}C-labeled XDE-208 concentrations in sediment, a 10-day NOEC is 0.0488 mg XDE-208 TRR/kg, corrected 10-day NOEC is 0.036 mg XDE-208/kg sediment.

The regulatory endpoints are 10-day LC_{50} 0.119 mg XDE-208/kg sediment and 10-day NOEC 0.036 mg XDE-208/kg sediment (based on corrected mean measured concentrations in sediment).

Study 4: Chronic toxicity to the sediment dwelling invertebrate Chironomus riparius (Sulfoxaflor DAR, Volume 3 - B.9.2.1.3.viii)

Method	Test organism	Test design	Results (mg a.s./L)			Remarks	Reference
			Endpoints	NOEC [mg/L]	LC_{50}/EC_{50} [mg/L]		

OECD 219	*Chironomus riparius*	chronic, 28d, spiked water, static	survival emergence	0.0384	-		Gerke, A. 2009

Citation: Gerke, A. (2009): Sulfoxaflor: Chronic Toxicity in Whole Sediment to Freshwater Midge, *Chironomus riparius*. ABC Laboratories, Columbia, Missouri, ABC 63674. Dow AgroSciences unpublished report, Study Number 080072. 19 May 2009.

Guidelines: OECD 219

GLP compliance: Yes.

Test material:

Test item: Sulfoxaflor and ^{14}C-Sulfoxaflor (X11859293)

Purity: Sulfoxaflor: 95.6% w/w

^{14}C-Sulfoxaflor: 99.7% w/w, 45.2 mCi/mmol

Description: White solid

Lot No./Batch No.: Sulfoxaflor: TSN003725-0001, E2162-34

^{14}C-Sulfoxaflor: INV027474-0001, XS9-37562-34

Material and methods:

A 28 day test was performed with nominal overlying water concentrations of 0 (control), 0.00157, 0.00313, 0.00625, 0.0125, 0.0250, 0.0500, and 0.100 mg a.s./L. The test material consisted of non-radiolabelled Sulfoxaflor mixed with ^{14}C-Sulfoxaflor radiolabel to act as a tracer in the ratio of 5.79 unlabeled:2.21 labelled. Approximately 200 g (approximately 2 cm sediment depth) of formulated sediment, prewetted with dilution water at approximately 35% of dry weight, was added to each replicate test chamber. A 600 mL volume of dilution water (approximately 10 cm) was carefully added to the test chambers and a plastic deflector was used during the water addition to minimize the disturbance of the sediment. Four replicate test chambers were prepared for the biological parameters. A total of four additional replicate chambers were prepared for the various analyses of the overlying water, pore water, and sediment samples. The test chambers were prepared eight days prior to study initiation. After the test chambers were set-up, they were inoculated at test initiation with a concentrated green algae [*Pseudokirchneriella subcapitata* (previously known as *Selenastrum capricornutum*)] solution in order to provide an initial food source for the larvae. A 0.00800 mg/mL primary stock solution was prepared by weighing a total of 2.21 mg of ^{14}C-labeled Sulfoxaflor and a total of 5.79 mg of non-radio labelled Sulfoxaflor into a 1,000-mL glass volumetric flask and bringing the flask to volume with dilution water. At the study initiation, aliquots of the primary stock solution were added using a pipette, and the overlying water was gently stirred to minimally disturb the sediment.

One day prior to study initiation, i.e., addition of the test substance to overlying water, a total of 20 midge larvae were added to each vial in a set of labelled containers. Each container was randomly assigned to a treatment replicate by a computer-generated random number table. The individuals

within a container were released into each biological replicate and the termination analytical replicates. There were four biological replicates per treatment level, resulting in 80 midge per test treatment. Aeration was provided at an initial rate of 60-100 bubbles per minute to each test chamber through a glass pipette. The pipette was inserted such that the tip was two to three centimetres from the sediment surface. Observations of the biological replicates were recorded at least every other day during the initial 13 days of the exposure and daily thereafter. Any abnormal activity (i.e., sediment avoidance, inactivity, etc.) was noted, if observed. The larvae were fed on a daily basis. Daily emergence observations (i.e., adult flies retained within the emergence traps) were recorded. Evidence of emergence was noted by the presence of exuviae as well as adults. Where possible, the adult flies observed in the emergence traps were identified and enumerated by gender and also for total emergence. If an exuviae was present but there was no adult fly present (i.e., escaped) or if there was a greater number of exuviae present than was accounted for by the number of emergent adults, then these missing adults were recorded to be of an unknown gender. Although gender could not be determined in the missing emergent adults, these organisms were still included in the total development rate calculation. At test termination, the sediments were sieved and surviving larvae or pupae, if any, were retained by the mesh and were recorded. These organisms were included with the total number of emergent adults to determine the 28-day survival values for each treatment level.

Measurements of temperature, dissolved oxygen concentration, and pH of the overlying water were measured at test initiation and at least weekly in each replicate test chamber. On days 0 and 28, composite samples of overlying water were taken from each biological replicate for measurement of total hardness and ammonia concentrations.

Statistical analysis: Statistical analysis of the concentration versus effect data was performed using SAS software. The no-observable effect concentration (NOEC) values for emergence, survival, time to emergence, and development rate were determined by using a one-way analysis of variance (ANOVA), followed by a Dunnett’s test for determination of significance. The Dunnett's test was conducted at the 0.05 level of significance with the alternate hypothesis being that response in the treatment had been reduced in comparison to the control. Prior to the ANOVA and Dunnett’s tests, a Shapiro-Wilk’s test and a Levene’s test were conducted to test for normality and homogeneity of variance, respectively. Data for male, female, and total development rate, and female and total emergence time, were normally distributed and the variances were homogeneous, so parametric analyses were performed. Survival, emergence and male emergence time data were not normally distributed and the variances were not homogeneous, so non-parametric analyses were performed. The NOEC was the highest concentration tested that was not statistically different from the control.

To determine that exposure to Sulfoxaflor did not differentially inhibit the development of the midge based upon specific gender, each gender was analyzed separately for statistical significance. The genders were also pooled for the total adult development rate data which was analyzed to determine statistical differences between the control and the treatment data.

Estimates of the effective concentration (EC50) value and the 95% confidence limits were estimated using the probit method and Trimmed Spearman-Karber method. When the P value for Goodness of Fit was >0.05 and there was no other evidence of questionable convergence, the probit method was selected for reporting. When this criterion was not achieved, the Trimmed Spearman-Karber method was selected for reporting.

The adult emergence ratios were calculated by dividing the total number of emergent adult midges by the initial number of larvae added to each replicate test chamber. The gender ratio was determined by dividing the total number of emergent males by the total number of emergent females. The percent emergence values were calculated by multiplying the emergence ratio by 100. The development rate for male, female, and total adult emergence was calculated by the following

equation:

$$\text{Mean Development} = \sum_{i=1}^{m} \frac{f_i x_i}{n_e}$$

where:

i = index of inspection interval

m = maximum number of inspection intervals

fi = number of midges emerged in the inspection interval i

ne = total number of midges emerged at the end of experiment

xi = development rate of the midges emerged in interval i

$$x_i = 1 \Big/ \left(\text{day}_i - \frac{l_i}{2} \right)$$

where:

dayi = inspection day (days since application)

li = length of inspection interval

Deviations to the study plan:

1. Upon test termination, it was noted that one replicate had more than 20 midge assigned to the test chamber. Control replicate B contained 22 midge.
   Reason: Extra midge were inadvertently added to the test chambers.

   Impact on study: None. This deviation does not adversely affect the study integrity or the interpretation of the results.

2. The stock solution of the test substance was prepared in dilution water not ABC reagent water.
   Reason: Technical oversight.

   Impact on study: None. The dilution water used was the same water added as the overlying water while setting up the test chambers. The analytical results at study initiation show there was not a solubility problem using this dilution water since the recoveries were between 105 and 112% of the nominal concentrations. This deviation does not adversely affect the study integrity or the interpretation of the results.

Results

All chemical and physical parameters for the 28-day study were within expected ranges. The overall temperature range measured in the overlying water was between 18.9 and 20.3ºC. Dissolved oxygen measurements ranged from 5.9 to 8.1 mg/L (68 to 92% of air saturation). The pH ranged from 8.0 to 8.4 in the overlying water in all concentration levels, which meets the acceptability criterion of the guidance document for this parameter. Total hardness values for the overlying water throughout the

study ranged from 204 to 236 mg $CaCO_3$/L.

Overlying water, interstitial (pore) water, and sediment were analyzed for total radioactive residues (TRR) by liquid scintillation counting (LSC) of duplicate samples. Duplicate samples were collected from each control or test substance treatment at test initiation and test termination. Overlying water was also analyzed for XDE-208 and the metabolite X11719474 using a high performance liquid chromatographic/mass spectrometry (HPLC/MS/MS) system. The measured concentrations of ^{14}C-labeled XDE-208 within the overlying water samples are given in Table B.9.2.1.22. The general decrease in TRR concentrations of the overlying water during the test is likely due to the test substance being incorporated into the pore water and the sediment. The measured concentrations of ^{14}C-labeled XDE-208 within the porewater samples and whole sediment samples are given in Tables B.9.2.14 and B.9.2.15. The measured concentrations of XDE-208 and X11719474 by HPLC/MS/MS analysis within the overlying water samples are given in Table B.9.2.16. The parent concentrations of XDE-208 indicate a more rapid decrease than the decrease in the TRR concentration in the overlying water, which suggests that the test substance may be incorporated into the pore water or sediments as well as metabolizing to X11719474 over the 28-day test exposure. This conclusion was supported when the concentration of X11719474 was added to the concentration of XDE-208 in the termination analytical samples and the result was approximately equivalent to the TRR concentrations.
All biological response evaluations were calculated based on initial and mean measured ^{14}C-labeled XDE-208 concentrations in the overlying water.

Table 5.4.4.Study 4.1 (DAR Table B.9.2.1.22) Analytical results from analysis of overlying water samples for XDE-208 total radioactive residues (TRR).

Overlying Water Nominal Concentration (mg a.i./L)	Mean Measured Concentrations (mg TRR/L) (percent nominal)		
	Day 0	Day 28	Mean
0 (control)	<MQL	<MQL	<MQL
0.00157	0.00171 (109%)	0.00113 (72%)	0.00142 (90%)
0.00313	0.00344 (110%)	0.00228 (73%)	0.00286 (91%)
0.00625	0.00704 (113%)	0.00504 (81%)	0.00604 (97%)
0.0125	0.0133 (106%)	0.00912 (73%)	0.0112 (90%)
0.0250	0.0269 (108%)	0.0181 (72%)	0.0225 (90%)
0.0500	0.0526 105%)	0.0383 (77%)	0.0455 (91%)
0.100	0.111 (111%)	0.0787 (79%)	0.0949 (95%)
MQL:	Day 0: 0.0000363 mg TRR/L; Day 28: 0.0000380 mg TRR/L		

Table 5.4.4.Study 4.2 (DAR Table B.9.2.1.23) Analytical results from analysis of pore water samples for XDE-208 total radioactive residues (TRR).

Overlying Water Nominal Concentration (mg a.i./L)	Mean Measured Concentrations (mg TRR/L)		
	Day 0	Day 28	Mean
0 (control)	<MQL	<MQL	<MQL
0.00157	0.0000965	0.000915	0.000506
0.00313	0.000234	0.00194	0.00109
0.00625	0.000343	0.00402	0.00218
0.0125	0.000745	0.00840	0.00457
0.0250	0.00179	0.0173	0.00955
0.0500	0.00275	0.0351	0.0189
0.100	0.00754	0.0655	0.0365
MQL:	Day 0: 0.0000391 mg TRR/L; Day 28: 0.0000480 mg TRR/L		

Table 5.4.4.Study 4.3 (DAR Table B.9.2.1.24) Analytical results from analysis of whole sediment samples for XDE-208 total radioactive residues (TRR).

Overlying Water Nominal Concentration (mg a.i./L)	Mean Measured Concentrations (mg TRR/kg d.w. sediment)			
	Day 0	Day 28	Mean	Other Parameters
0 (control)	<MQL	<MQL	<MQL	Type: Artificial OECD
0.00157	0.000704	0.00144	0.00107	Total organic matter (%): 5 % sphagnum peat
0.00313	<MQL	0.00335	0.00182	Clay (%): 20
0.00625	<MQL	0.00660	0.00345	Sand (%): 75
0.0125	0.000842	0.0129	0.00687	Silt (%): Not determined
0.0250	0.00175	0.0264	0.0141	
0.0500	0.00261	0.0527	0.0277	
0.100	0.00796	0.0984	0.0532	
MQL:	Day 0: 0.000594 mg TRR/kg d.w. sediment; Day 28: 0.000632 mg TRR/kg d.w. sediment			

Table 5.4.4.Study 4.4 (DAR Table B.9.2.1.25) Analytical results from analysis of overlying water samples for XDE-208 and metabolite X11719474 by confirmatory LC/MS/MS.

Overlying Water Nominal Concentration of XDE-208 (mg/kg dry sediment)	Mean Measured Concentrations (mg/L) (percent nominal)		
	Day 0	Day 28	Day 0-28 Mean
XDE-208			
0 (control)	<MQL	<MQL	<MQL
0.1	0.108 (108%)	0.0502 (50%)	0.0791 (79%)
X11719474			
0 (control)	<MQL	<MQL	<MQL
0.1	<MQL	0.0269	0.0166[a]
MQL for XDE-208:	Day 0 and Day 28: 0.0125 mg/L		
MQL for X11719474:	Day 0 and Day 28: 0.0125 mg/L		
[a] Mean calculation used ½ of the MQL for the Day 0 value.			

A summary of the total adult emergence, emergence ratio, and percent emergence is presented in Table B.9.2.1.26. Emergence was observed in the control on day 14 with a single adult female observed on day 28. This one female did not adversely affect the validity of the study since the majority of the control emergence (>95%) occurred between days 15 and 23 after addition of the test substance. In the treatments, emergence was observed starting on day 14 with the last emerged midge observed on day 27. The gender ratio for the control was 1.1 males to each female. The male to female gender ratio for the treatments ranged from 0.64 in the 0.0269 mg TRR/L treatments to 1.3 in the 0.111 mg TRR/L treatment. There was not a concentration dependent effect of the test substance on the observed gender ratios. Therefore statistical analysis of the emergence rates was based upon total adult emergence.

A summary of the development rates, survival, and observations is presented in Table B.9.2.1.27. Calculated effects concentrations for emergence and development rate of the *Chironomus riparius*, exposed to XDE-208, are presented in Table B.9.2.1.28.

Table 5.4.4.Study 4.5 (DAR Table B.9.2.1.26) Effect of XDE-208 on adult emergence and development rate of *Chironomus riparius*

Initial Measured Overlying Water Concentration (mg TRR/L)	Sex of emerged midge	Adult Emergence (by Day 28)				
		Rep 1	Rep 2	Rep 3	Rep 4	Mean of all replicates
Control	**% Emerged**	**80**	**100**	**85**	**100**	**91**
	M Dev. Rate	0.0628	0.0544	0.0630	0.0570	0.0593
	F Dev. Rate	0.0538	0.0523	0.0551	0.0476	0.0522
	T Dev. Rate	0.0599	0.0525	0.0590	0.0514	0.0557
	Mean F Emerge Time	19.2	19.8	18.8	21.7	19.9
	Mean M Emerge Time	16.6	19.2	16.5	18.1	17.6
0.00171	**% Emerged**	**85**	**85**	**65**	**85**	**80**
	M Dev. Rate	0.0569	0.0621	0.0610	0.0610	0.0603
	F Dev. Rate	0.0546	0.0560	0.0601	0.0594	0.0575
	T Dev. Rate	0.0558	0.0588	0.0608	0.0605	0.0590
	Mean F Emerge Time	19.1	18.6	17.3	17.5	18.1
	Mean M Emerge Time	18.4	16.8	17.3	17.1	17.4
0.00344	**% Emerged**	**85**	**90**	**80**	**65**	**80**
	M Dev. Rate	0.0611	0.0588	0.0631	0.0557	0.0597
	F Dev. Rate	0.0540	0.0561	0.0509	0.0518	0.0532
	T Dev. Rate	0.0568	0.0571	0.0590	0.0536	0.0566
	Mean F Emerge Time	19.3	19.7	20.2	20.1	19.8
	Mean M Emerge Time	17.0	18.0	16.6	18.8	17.6
0.00704	**% Emerged**	**85**	**85**	**95**	**90**	**89**
	M Dev. Rate	0.0578	0.0582	0.0566	0.0610	0.0584
	F Dev. Rate	0.0534	0.0530	0.0521	0.0572	0.0539
	T Dev. Rate	0.0552	0.0560	0.0534	0.0586	0.0558
	Mean F Emerge Time	19.7	19.5	20.0	18.2	19.4
	Mean M Emerge Time	17.9	17.9	18.6	17.0	17.9
0.0133	**% Emerged**	**70**	**85**	**85**	**80**	**80**
	M Dev. Rate	0.0607	0.0576	0.0694	0.0632	0.0627
	F Dev. Rate	0.0604	0.0536	0.0497	0.0487	0.0531
	T Dev. Rate	0.0606	0.0552	0.0567	0.0516	0.0560
	Mean F Emerge Time	17.3	19.5	20.7	21.2	19.7
	Mean M Emerge Time	17.2	18.0	15.0	16.3	16.6
0.0269	**% Emerged**	**95**	**75**	**75**	**80**	**81**

Initial Measured Overlying Water Concentration (mg TRR/L)	Sex of emerged midge	Adult Emergence (by Day 28)				
		Rep 1	**Rep 2**	**Rep 3**	**Rep 4**	**Mean of all replicates**
	M Dev. Rate	0.0524	0.0581	0.0586	0.0583	0.0569
	F Dev. Rate	0.0523	0.0573	0.0541	0.0508	0.0536
	T Dev. Rate	0.0523	0.0585	0.0561	0.0537	0.0552
	Mean F Emerge Time	20.0	18.3	19.1	20.4	19.5
	Mean M Emerge Time	20.0	18.0	18.0	18.0	18.5
0.0526	**% Emerged**	**85**	**85**	**90**	**60**	**80**
	M Dev. Rate	0.0595	0.0612	0.0520	0.0557	0.0571
	F Dev. Rate	0.0489	0.0541	0.0459	0.0548	0.0509
	T Dev. Rate	0.0575	0.0561	0.0483	0.0552	0.0543
	Mean F Emerge Time	21.0	19.3	22.8	18.9	20.5
	Mean M Emerge Time	17.6	17.0	20.1	18.8	18.4
0.111	**% Emerged**	**70**	**60**	**70**	**80**	**70***
	M Dev. Rate	0.0555	0.0582	0.0567	0.0584	0.0572
	F Dev. Rate	0.0490	0.0514	0.0528	0.0526	0.0515
	T Dev. Rate	0.0511	0.0559	0.0544	0.0571	0.0546
	Mean F Emerge Time	21.1	20.0	19.7	21.0	20.5
	Mean M Emerge Time	18.8	17.9	18.8	17.9	18.4

M=Male, F=Female, T=Total, Dev. Rate = Development Rate, TRR = Total Radioactive Residues
* Statistically significant (p = 0.05) effect as compared to the controls.

Table 5.4.4.Study 4.6 (DAR Table B.9.2.1.27) Development rates and overall survival data at the termination of the 28-day exposure with the *Chironomus riparius* to XDE-208

Initial Measured Overlying Water Concentration (mg TRR/L)	REP	Male Rate	Female Rate	Total Rate [a]	Percent Survival	Observations [b]
Control	A	0.0628	0.0538	0.0599	80	16 E
	B	0.0544	0.0523	0.0525	100	22 E
	C	0.0630	0.0551	0.0590	85	17 E
	D	0.0570	0.0476	0.0514	100	20 E
	Mean	0.0593	0.0522	0.0557	91	---
0.00171	A	0.0569	0.0546	0.0558	85	17 E
	B	0.0621	0.0560	0.0588	85	17 E
	C	0.0610	0.0601	0.0608	65	13 E
	D	0.0610	0.0594	0.0605	85	17 E
	Mean	0.0603	0.0575	0.0590	80	---
0.00344	A	0.0611	0.0540	0.0568	85	17 E
	B	0.0588	0.0561	0.0571	90	18 E
	C	0.0631	0.0509	0.0590	80	16 E
	D	0.0557	0.0518	0.0536	65	13 E
	Mean	0.0597	0.0532	0.0566	80	---
0.00704	A	0.0578	0.0534	0.0552	85	17 E
	B	0.0582	0.0530	0.0560	85	17 E
	C	0.0566	0.0521	0.0534	95	19 E
	D	0.0610	0.0572	0.0586	90	18 E
	Mean	0.0584	0.0539	0.0558	89	---
0.0133	A	0.0607	0.0604	0.0606	70	14 E
	B	0.0576	0.0536	0.0552	85	17 E
	C	0.0694	0.0497	0.0567	85	17 E
	D	0.0632	0.0487	0.0516	80	16 E
	Mean	0.0627	0.0531	0.0560	80	---
0.0269	A	0.0524	0.0523	0.0523	95	19 E
	B	0.0581	0.0573	0.0585	75	15 E
	C	0.0586	0.0541	0.0561	75	15 E
	D	0.0583	0.0508	0.0537	80	16 E
	Mean	0.0569	0.0536	0.0552	81	---
0.0526	A	0.0595	0.0489	0.0575	85	17 E
	B	0.0612	0.0541	0.0561	85	17 E
	C	0.0520	0.0459	0.0483	90	18 E
	D	0.0557	0.0548	0.0552	60	12 E
	Mean	0.0571	0.0509	0.0543	80	---
0.0111	A	0.0555	0.0490	0.0511	70	14 E
	B	0.0582	0.0514	0.0559	60	12 E
	C	0.0567	0.0528	0.0544	70	14 E
	D	0.0584	0.0526	0.0571	80	16 E
	Mean	0.0572	0.0515	0.0546	70	---

[a] Mean development rate of all adults (males, females, unknown gender) emerged during the study.
[b] Key to Observations: E = Number emerged (alive).

Table 5.4.4.Study 4.7 (DAR Table B.9.2.1.28) Calculated effects concentrations for emergence and development rate of the *Chironomus riparius*, exposed to XDE-208

Based on Initial Measured Concentrations:

Biological Parameter	NOEC [a]	LOEC [a]	LC_{50}
Survival	0.0526 mg TRR/L	0.111 mg TRR/L	>0.111 mg TRR/L
Emergence	0.0526 mg TRR/L	0.111 mg TRR/L	>0.111 mg TRR/L
Emergence Time (Male)	0.111 mg TRR/L	---	---
Emergence Time (Female)	0.111 mg TRR/L	---	---
Emergence Time (Total)	0.111 mg TRR/L	---	---
Development Rate (Male)	0.111 mg TRR/L	---	---
Development Rate (Female)	0.111 mg TRR/L	---	---
Development Rate (Total)	0.111 mg TRR/L	---	---

Based on Mean Measured Concentrations:

Biological Parameter	NOEC [a]	LOEC [a]	LC_{50}
Survival	0.0455 mg TRR/L	0.0949 mg TRR/L	>0.0949 mg TRR/L
Emergence	0.0455 mg TRR/L	0.0949 mg TRR/L	>0.0949 mg TRR/L
Emergence Time (Male)	0.0949 mg TRR/L	---	---
Emergence Time (Female)	0.0949 mg TRR/L	---	---
Emergence Time (Total)	0.0949 mg TRR/L	---	---
Development Rate (Male)	0.0949 mg TRR/L	---	---
Development Rate (Female)	0.0949 mg TRR/L	---	---
Development Rate (Total)	0.0949 mg TRR/L	---	---

[a] No-observable-effect concentration and lowest-observable-effect concentration.

"---" indicates not calculated

Reliability of the study

The reported study is GLP compliant and conducted to a standard study protocol. Two deviations to the study plan were noted, but they are considered minor and they are not supposed to result in any significant effects on the study. The test results are in compliance with the guideline's validity criteria. The study is acceptable for regulatory use.

Because the confirmatory analysis by LC/MS/MS (Table Table B.9.2.16) of concentrations of XDE-208 in overlying water showed that the levels of XDE-208 dropped by approximately 50% over the course of the 28 day exposure, the suggested adjustment of the endpoints to reflect the mean measured concentration of XDE-208 parent, rather than using the initial measured Total Radioactive Residue (TRR) concentrations which included residues of metabolite X11719474. The mean measured recovery of XDE-208 in the 0.1 mg/L nominal treatment level was 73% (28 day mean measured 0.079 mg a.i./L / initial measured 0.108 mg a.s/L x 100% = 73%). With this value,

the endpoints determine above as initial measured TRR concentrations can be corrected by a factor of 73% to provide endpoints expressed as mean measured XDE-208 concentrations. Based on the total adult emergence and corrected mean measured XDE-208 concentrations, the estimated 28-day EC_{50} value was >0.081 mg a.i./L, the highest concentration tested. Based on survival and corrected mean measured concentrations, the estimated 28-day LC_{50} value was >0.081 mg a.i./L, the highest concentration tested. Based on no statistically significant effects on male, female, and total adult average emergence time and development rate and corrected mean measured concentrations, the 28-day no observed effect concentration (NOEC) was 0.081 mg a.i./L for emergence time and development rate. Based on survival and emergence and corrected mean measured concentrations, the 28-day NOEC was 0.0384 mg a.i./L and the LOEC was 0.081 mg a.i./L.

The correction of the toxicity endpoints suggested was accepted.

Based on initial measured ^{14}C-labeled XDE-208 concentrations in overlying water, a 28-day LC_{50} and EC_{50} is >0.111 XDE-208 TRR/L, corrected 28-day LC_{50} and EC_{50} is >0.081 mg XDE-208/L. Based on initial measured ^{14}C-labeled XDE-208 concentrations in overlying water, a 28-day NOEC is 0.0526 mg XDE-208 TRR/|L, corrected 28-day NOEC is 0.0384 mg XDE-208/L.

The regulatory endpoints are 28-day LC_{50} and EC_{50} >0.081 mg XDE-208/L and 28-day NOEC 0.0384 mg XDE-208/L (based on corrected initial measured concentrations in overlying water).

5.4.5 Summary and discussion of the aquatic toxicity

The submitted toxicity studies indicate that Sulfoxaflor exhibits low acute toxicity to fish, freshwater crustaceans (*Daphnia*), oysters, algae and aquatic vascular plants, while it is of particularly high acute and chronic toxicity to the midge larvae *Chironomus* (a sediment-dwelling insect) and the mysid shrimp *Americamysis* (a saltwater free-swimming crustacean). The most sensitive organism to the effects of Sulfoxaflor appeared to be the sediment-dweller *Chironomus*.

Two types of acute toxicity studies were submitted for *Chironomus dilutus*: an acute 96-hour spiked-water test, giving the acute toxicity value of LC_{50} = 0.622 mg a.s./L, and an acute 10-day whole sediment exposure test, giving the acute toxicity value of LC_{50} = 0.119 mg a.s./kg sediment dry weigth. For classification and labelling, the spiked-water test with LC_{50} = 0.622 mg a.s./L has been selected. The lowest chronic toxicity value of NOEC = 0.0384 mg a.s./L was produced in an artificial sediment 28-day emergence test with *Chironomus riparius*, exposed via the overlying water. Laboratory chronic toxicity studies indicate Sulfoxaflor to be slightly toxic to *Daphnia*, and to exhibit slight effects on growth in a long-term early-life-stage toxicity test in fathead minnow (*Pimephales promelas*), but stronger effects on growth in an early life stage toxicity study in sheepshead minnow (*Cyprinodon variegatus*).

5.5 Comparison with criteria for environmental hazards (sections 5.1 – 5.4)

Summary of Environmental Hazards:

Degradation:

In the aquatic environment Sulfoxaflor was demonstrated to be hydrolytically and photolytically stable in the whole range of environmentally relevant pH (5-9) – for the aqueous hydrolysis

DT50 >1000 days, for the direct aqueous photolysis DT50 = 489 days and for the indirect aqueous photolysis DT50 = 224 days. It can be therefore concluded that in none of the abiotic processes at least 70% of sulfoxaflor degraded within 28 days, so this compound cannot be considered rapidly degradable in abiotic processes in water. In the study on ready biodegradability it was demonstrated that within 28 days only up to 2.5% of it underwent mineralization, while at the same time the reference compound was mineralized completely. Therefore sulfoxaflor does not meet biodegradability criterion, i.e at least 70% mineralization within 28 days. This observation was confirmed by the results of the study on the degradation in biologically viable aquatic system (water/sediment study), in which only up to 1.6% of it was mineralized by the end of the study (on day 88th). On this basis it can be stated that sulfoxaflor is not ready biodegradable. Finally, in the same study on the degradation in biologically viable aquatic system (water/sediment study) it was demonstrated that the average (geomean) DT50 for this compound was 57.08 days (the whole system value), therefore within 28 days much less than 70% of it undergoes biological degradation. As a result it can be stated that sulfoxaflor is not rapidly biologically degradable.

The final conclusion on the degradation of sulfoxaflor in the environment is that this compound is neither readily biodegradable nor rapidly degradable in the environment.

Bioaccumulation:

Analysing the physical-chemical properties of Sulfoxaflor, as well as its sorptive behaviour the compound has a very low affinity to organic compounds in general and lipids in particular.

Its solubility in water (unbuffered pure water at C = 20°C) is high for an organic compound - 670.3 mg/L. The Log Pow (20°C (99.7%)) is at pH 5: Log Pow= 0.806; at pH 7: Log Pow= 0.802; and at pH 9: Log Pow= 0.799, indicating that Sulfoxaflor has low or even very low affinity to lipids and other non-polar organic compounds (hence low expected bioaccumulation potential).

The results of the water/sediment studies indicate that this compound should be expected to occur mainly in the water phase. This is confirmed, although indirectly, by the soil adsorption constants. As a consequence, Sulfoxaflor exhibits low bioaccumulation potential in either aquatic plants or aquatic animals because of the low affinity to lipids and, probably lignins. The same concerns, X11719474 and other major metabolites.

The log Pow of Sulfoxaflor was found to be 0.799 - 0.806 at 20°C. Hence no bioconcentration study is demanded. There was no experimental data and no measured bioaccumulation data are available.

Based on the measured log POW (0.799 - 0.806 at 20 °C) XDE-208 is considered to have a low bioaccumulation potential.

Aquatic Toxicity:

The submitted toxicity studies indicate that Sulfoxaflor exhibits low acute toxicity to fish, freshwater crustaceans (Daphnia), oysters, algae and aquatic vascular plants, while it is of

particularly high acute and chronic toxicity to the midge larvae Chironomus (a sediment-dwelling insect) and the mysid shrimp Americamysis (a saltwater free-swimming crustacean). The most sensitive organism to the effects of Sulfoxaflor appeared to be the sediment-dweller Chironomus.

Two types of acute toxicity studies were submitted for Chironomus dilutus: an acute 96-hour spiked-water test, giving the acute toxicity value of LC50 = 0.622 mg a.s./L, and an acute 10-day whole sediment exposure test, giving the acute toxicity value of LC50 = 0.119 mg a.s./kg sediment dry weigth. For classification and labelling, the spiked-water test with LC50 = 0.622 mg a.s./L has been selected.

The lowest chronic toxicity value of NOEC = 0.0384 mg a.s./L was produced in an artificial sediment 28-day emergence test with Chironomus riparius, exposed via the overlying water. This value was determined based on survival and emergence and on the correction of the initial measured TRR concentrations corrected by a factor of 73% to provide endpoints expressed as mean measured XDE-208 concentrations. The initial measured 14^{C}-labeled XDE-208 concentrations, a 28-day NOEC, is 0.0526 mg XDE-208 TRR/|L which provides a corrected 28-day NOEC of 0.0384 mg XDE-208/L. Laboratory chronic toxicity studies indicate Sulfoxaflor to be slightly toxic to Daphnia, and to exhibit slight effects on growth in a long-term early-life-stage toxicity test in fathead minnow (Pimephales promelas), but stronger effects on growth in an early life stage toxicity study in sheepshead minnow (Cyprinodon variegatus).

Comparison with the criteria:

Degradation:

In the study on ready biodegradability it was demonstrated that within 28 days only up to 2.5% of the active substance underwent mineralization, while at the same time the reference compound was mineralized completely. Other scientific evidence indicates that at least 70% mineralization does not occur within 28 days. Therefore sulfoxaflor does not meet biodegradability criterion, i.e at least 70% mineralization within 28 days.

Bioaccumulation:

Based on the measured log POW (0.799 - 0.806 at 20 °C) sulfoxaflor is considered to have a low bioaccumulation potential. Hence no bioconcentration study is demanded. There was no experimental data and no measured bioaccumulation data are available.

The cut off value of log ≥ 4 is uded to identify substances with real potential to bioconcentrate. The measured log POW is (0.799 - 0.806 at 20 °C) therefore sulfoxaflor is considered to have a low bioaccumulation potential.

Aquatic Toxicity:

For consideration of classification and labelling in terms of the acute aquatic hazard, the spiked-water test with LC50 = 0.622 mg a.s./L for Chironomus dilutus has been selected as this is the lowest acute toxicity value of the active substance for the most sensitive tested aquatic organism. The criteria for acute aquatic hazard indicates that if the value is ≤ 1 mg/l then classification for acute aquatic hazard category 1 will apply.

For consideration of classification and labelling in terms of the chronic aquatic hazard, the lowest chronic toxicity value of NOEC = 0.0384 mg a.s./L was produced in an artificial sediment 28-day emergence test with Chironomus riparius, exposed via the overlying water. In addition, the active substance is not readily biodegradable and not rapidly biodegradable. The criteria for chronic (long-term) aquatic hazard indicates that if the value is ≤ 1 mg/l and the substance is not rapidly

degradable then classification for chronic aquatic hazard category 1 will apply.

Justification for the proposal:

H400 follows from the lowest acute toxicity value of the active substance for the most sensitive tested aquatic organism with LC_{50} < 1 mg a.s./L (*Chironomus dilutus* LC_{50} = 0.622 mg a.s./L, Gerke, 2008d). A M-factor of 1 is applicable based on 0.1 < LC_{50} ≤1 mg a.s./l.

H410 follows from the lowest chronic toxicity value of the active substance for the most sensitive tested aquatic organism with an NOEC ≤ 1 mg a.s./L (*Chironomus riparius* NOEC = 0.0384 mg/L, Gerke, 2009) and the fact that the active substance is not readily biodegradable and not rapidly biodegradable. A M-factor of 1 is applicable based on 0.01 < NOEC ≤ 0.1 mg/l.

GHS09 Pictogram is required for 'Aquatic acute 1' and 'Aquatic chronic 1' category substance.

Signal word 'Warning' is required for 'Aquatic acute 1' and 'Aquatic chronic 1' category substance.

The statements P273, P391 and P501 follow a general precautionary approach for dangerous substances.

Conclusion of environmental classification according to Regulation EC 1272/2008:

Pictogram: GHS09 Signal word: Warning Aquatic acute 1, M = 1, H400: Very toxic to aquatic life. Aquatic chronic 1, M = 1, H410: Very toxic to aquatic life with long lasting effects.

Justification for the proposal:

R50 follows from the lowest acute toxicity value of the active substance for the most sensitive tested aquatic organisms with LC_{50} < 1 mg a.s./L (*Chironomus dilutus*: LC_{50} = 0.622 mg a.s./L, Gerke, 2008d).

R53 follows from the fact that the active substance is not readily biodegradable.

The safety phrases S60 and S61 have to be applied based on the proposed R50/53.

Conclusion of environmental classification and labelling according to Directive 67/548/EEC:

N Dangerous for the environment. R50 Very toxic to aquatic organisms. R53 May cause long term effects in the environment. S60 This material and its container must be disposed of as hazardous waste. S61 Avoid release to the environment. Refer to special instructions/Safety Data Sheet.

5.6 Conclusions on classification and labelling **for environmental hazards (sections 5.1 – 5.4)**

Conclusion of environmental classification according to Regulation EC 1272/2008:

The active substance fulfils the criteria for classification for aquatic environmental hazard based on the CLP Regulation.

Pictogram:	**GHS09**
Signal word:	**Warning**
Classification categories:	**Aquatic acute 1**, **Aquatic chronic 1 ~**
Hazard statements:	**H400: Very toxic to aquatic life, H410: Very toxic to aquatic life with long lasting effects.**
M-factor:	**Acute M-factor 1 and Chronic M-factor 1**
Precautionary statements:	**P273 Avoid release to the environment**, **P391 Collect spillage**, **P501 Dispose of contents/ container to ... (in accordance with local/ regional/ national/ international regulation (to be specified))**

Conclusion of environmental classification and labelling according to Directive 67/548/EEC:

The active substance fulfils the criteria for classification as N, R 50/53 according to Directive 67/548/EEC.

Hazard symbol:		**Dangerous for the environment**
Risk phrases:	**R 50/53**	**Very toxic to aquatic organisms, may cause long term adverse effects in the aquatic environment**
Safety phrases:	**S60**	**This material and its container must be disposed of as hazardous waste**
	S61	**Avoid release to the environment. Refer to special instructions/safety data sheets**

6 OTHER INFORMATION

No further information

7 REFERENCES

Section 1-3

Author(s)	Year	Title Source (where different from the Company), Company, Report Number, GLP or GEP status (where relevant), Published or not	
Madsen, S.	2009a	Determination of Color, Physical State, Odor, Melting Point and Decomposition Temperature of XDE-208 Pure Active Ingredient	
		Dow AgroSciences, Indianapolis, IN 46268, USA	
		DAS Report No.:	FAPC-G-09-15
		GLP/GEP (Y/N):	Y
		Published (Y/N):	N
Sarff, P.	2008a	XDE-208 TGAI: Determination of Density for Solids	
		ABC Laboratories Inc, Columbia, MO 65202; USA	
		DAS Report No.:	NAFST-08-024
		GLP/GEP (Y/N):	Y
		Published (Y/N):	N
Turner, B.	2009a	Determination of Vapour Pressure for XDE-208 PAI	
		Huntingdon Life Sciences Ltd., Cambridgeshire, England UK	
		DAS Report No.:	NAFST-08-72
		GLP/GEP (Y/N):	Y
		Published (Y/N):	N
Madsen, S.	2009b	Determination of Color, Odor, Physical State, Oxidizing and Reducing Action, Explodability, pH and Bulk Density of XDE-208 Technical Grade Active Ingredient	
		Dow AgroSciences, Indianapolis, IN 46268, USA	
		DAS Report No.:	FAPC-G-09-14
		GLP/GEP (Y/N):	Y
		Published (Y/N):	N
Turner, B.	2009b	Determination of Water Solubility for XDE-208	
		Huntingdon Life Sciences Ltd, Cambridgeshire, Enland, ULK	
		DAS Report No.:	NAFST-08-73
		GLP/GEP (Y/N):	Y
		Published (Y/N):	N

Author(s)	Year	Title Source (where different from the Company), Company, Report Number, GLP or GEP status (where relevant), Published or not	
Turner, B.	2009d	Determination of Octanol/Water Partition Coefficient for XDE-208 PAI by Shake Flask Method	
		Huntingdon Life Sciences Ltd, Cambridgeshire, England, UK	
		DAS Report No.:	NAFST-08-74
		GLP/GEP (Y/N):	Y
		Published (Y/N):	N

Author(s)	Year	Title Source (where different from the Company), Company, Report Number, GLP or GEP status (where relevant), Published or not	
Cathie, C.	2010a	Determination of Dissociation Constant of XDE-208 Using UV-Visible Spectrophotometry and Potentiometric Titration Techniques	
		Dow AgroSciences LLC, Indianapolis, IN 46268, USA	
		DAS Report No.:	10-003-G
		GLP/GEP (Y/N):	Y
		Published (Y/N):	N
Turner, B.	2009e	Determination of Surface Tension, Flammability, Self-Ignition Temperature and Oxidising Properties for XDE-208 TGAI	
		Huntingdon Life Sciences Ltd., Cambridgeshire, England, UK	
		DAS Report No.:	NAFST-08-75
		GLP/GEP (Y/N):	Y
		Published (Y/N):	N

Author(s)	Year	Title Source (where different from the Company), Company, Report Number, GLP or GEP status (where relevant), Published or not	
Turner, B.	2009f	Determination of Explosive Properties (Thermal and Friction Tests) for XDE-208 TGAI	
		Huntingdon Life Sciences Ltd., Cambridgeshire, England, UK	
		DAS Report No.:	NAFST-09-93
		GLP/GEP (Y/N):	Y
		Published (Y/N):	N

Section 4

Not applicable

Section 5

Fate and behaviour in the environment

Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant), Published or not
Yoder R. N., Liu D.	2010	Adsorption/Desorption of XDE-208 and Adsorption of Three XDE-208 Soil Metabolites.; Regulatory Sciences & Government Affairs, Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, Indiana 46268-1054, USA; Study report No. 080161, 04 January 2010; GLP: yes; Study not published.
Laughlin L. A.	2009	Hydrolysis of XDE-208 at pH 5, 7, and 9.; Regulatory Sciences & Government Affairs, Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, Indiana 46268-1054, USA; Study report No. 070102, 03 March 2009; GLP: yes; Study not published.
Ma M.	2011	Aqueous Photolysis of XDE-208 and X11719474 in pH 7 Buffer Under Xenon Light.; Regulatory Sciences & Government Affairs, Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, Indiana 46268-1054, USA; Study report No. 090073, 04 March 2011 (amended version of the report No. 47832283 of the 06 May 2010); GLP: yes; Study not published.
Yoder R. N.	2010	Natural Water Photolysis of XDE-208 and the X11719474 Metabolite under Xenon Light.; Regulatory Sciences & Government Affairs, Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, Indiana 46268-1054, USA; Study report No. 090088, 01 April 2010.

		GLP: yes; Study not published.
Fiel N.	2010	Ready Biodegradability of XDE-208 technical in a CO_2 Headspace Test. Institut für Biologische Analytik und Consulting IBACON GmbH, Arheilger Weg 17, 64380 Rossdorf, Germany, *for* Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, Indiana 46268-1054, USA; Study report No. 54631082; 18 May 2010; GLP: yes; Study not published.
Laughlin L. A., Adelfinskaya Y., Balcer J. L.	2010	Aerobic transformation of XDE-208 in Two European Aquatic Sediment Systems.; Regulatory Sciences & Government Affairs, Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, Indiana 46268-1054, USA; Study report No. 080138; 18 March 2010. GLP: yes; Study not published.
Weldenburg B. M., Boulton J. P	2010	Estimation of the Photochemical Oxidation Rate of XDE-208.; Regulatory Sciences & Government Affairs, Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, Indiana 46268-1054, USA; Study report No. 101449; 17 May 2010. GLP: no, not required (modelling study); Study not published.

Aquatic Toxicity

Author(s)	Year	Title Source (where different from the Company), Company, Report Number, GLP or GEP status (where relevant), Published or not
Gerke, A.	2008a	XDE-208: Acute Toxicity Test to the Rainbow Trout, *Oncorhynchus mykiss*, Determined Under Static Test Conditions ABC Laboratories, Inc., Columbia, Missouri, USA. DAS Report No.: 080064 GLP/GEP (Y/N): Y Published (Y/N): N
Gerke, A.	2008b	XDE-208: Acute Toxicity Test to the Bluegill Sunfish, *Lepomis macrochirus*, Determined Under Static Test Conditions ABC Laboratories, Inc., Columbia, Missouri, USA. DAS Report No.: 080065 GLP/GEP (Y/N): Y Published (Y/N): N
Gerke, A.	2008c	XDE-208: Acute Toxicity Test to the Common Carp, *Cyprinus carpio*, Determined Under Static Test Conditions ABC Laboratories, Inc., Columbia, Missouri, USA. DAS Report No.: 080066 GLP/GEP (Y/N): Y Published (Y/N): N
Gerke, A.	2008d	XDE-208: Acute Toxicity Test to the Sheepshead Minnow, *Cyprinodon variegatus*, Determined Under Static Test Conditoins ABC Laboratories, Inc., Columbia, Missouri, USA. DAS Report No.: 080067 GLP/GEP (Y/N): Y Published (Y/N): N

Author(s)	Year	Title Source (where different from the Company), Company, Report Number, GLP or GEP status (where relevant), Published or not
Boettcher, M., Wydra, V.	2009	Toxicity of XDE-208 Technical to Fathead Minnow (*Pimephales promelas*) in an Early-Life Stage Test Institut fur Biologische Analytik und Consulting IBACON GmbH, Rossdorf, Germany DAS Report No.: 080444 GLP/GEP (Y/N): Y Published (Y/N): N
Hicks, S.L.	2010	XDE-208: Early Life-Stage Toxicity Test with the Sheepshead Minnow, *Cyprinodon variegatus*, Under Flow-Through Conditions ABC Laboratories, Inc., Columbia, Missouri, USA. DAS Report No.: 101286 GLP/GEP (Y/N): Y Published (Y/N): N
Hicks, S.L.	2008a	XDE-208: Static Acute Toxicity Test with the Water Flea, *Daphnia magna* ABC Laboratories, Inc., Columbia, Missouri, USA. DAS Report No.: 080068 GLP/GEP (Y/N): Y Published (Y/N): N
Hicks, S.L.	2008b	XDE-208: Static Acute Toxicity Test with the Mysid Shrimp, *Americamysis bahia* ABC Laboratories, Inc., Columbia, Missouri, USA. DAS Report No.: 080069 GLP/GEP (Y/N): Y Published (Y/N): N
Kuhl, R., Wydra, V.	2009a	XDE-208 technical: Influence of XDE-208 Technical to *Daphnia magna* in a Reproduction Test Institut fur Biologische Analytik und Consulting IBACON GmbH, Rossdorf, Germany DAS Report No.: 080445 GLP/GEP (Y/N): Y Published (Y/N): N
Lehman, C	2010	XDE-208: Life-Cycle Toxicity Test of the Saltwater Mysid, *Americamysis bahia*, Conducted under Flow-Through Conditions ABC Laboratories, Inc., Columbia, Missouri, USA. DAS Report No.: 090534 GLP/GEP (Y/N): Y Published (Y/N): N
Dengler, D.	2009a	XDE-208: Testing of Effects of XDE-208 on the Single Cell Green Alga *Pseudokirchneriella subcapitata* in a Static 96 h Test Eurofins GAB GmbH, Niefern-Oschelbronn, Germany DAS Report No: 080439 GLP/GEP (Y/N): Y Published (Y/N): N
Dengler, D.	2009b	Testing of Effects of XDE-208 on the Marine Diatom *Skeletonema costatum* in a Static 96 h Test Eurofins GAB GmbH, Niefern-Oschelbronn, Germany DAS Report No.: 080440 GLP/GEP (Y/N): Y Published (Y/N): N

Author(s)	Year	Title Source (where different from the Company), Company, Report Number, GLP or GEP status (where relevant), Published or not
Dengler, D.	2009c	Testing of Effects of XDE-208 on the Blue-Green Alga *Anabaena flos-aquae* in a 96 h Static Test Eurofins GAB GmbH, Niefern-Oschelbronn, Germany DAS Report No.: 080442 GLP/GEP (Y/N): Y Published (Y/N): N
Dengler, D.	2009d	Testing of Effects of XDE-208 on the Diatom *Navicula pelliculosa* in a 96 h Static Test Eurofins GAB GmbH, Niefern-Oschelbronn, Germany DAS Report No.: 080441 GLP/GEP (Y/N): Y Published (Y/N): N
Kuhl, R. Wydra, V.	2009b	XDE-208 technical: Toxicity of XDE-208 technical to the Aquatic Plant *Lemna gibba* in a Semi-Static Growth Inhibition Test Institut fur Biologische Analytik und Consulting IBACON GmbH, Rossdorf, Germany DAS Report No.: 080443 GLP/GEP (Y/N): Y Published (Y/N): N
Hicks, S.L.	2008c	XDE-208: Effect on New Shell Growth of the Eastern Oyster (*Crassostrea virginica*) ABC Laboratories, Inc., Columbia, Missouri, USA. DAS Report No.: 080070 GLP/GEP (Y/N): Y Published (Y/N): N
Gerke, A.	2008e	XDE-208: Acute 96 Hour Toxicity to the Midge, *Chironomus dilutus*, Determined Under Static Test Conditions. ABC Laboratories, Inc., Columbia, Missouri, USA. DAS Report No.: 080362 GLP/GEP (Y/N): Y Published (Y/N): N
Gerke, A.	2008f	XDE-208: Whole Sediment 10 Day Acute Toxicity Test with Midge Larvae (*Chironomus dilutus*) ABC Laboratories, Inc., Columbia, Missouri, USA. DAS Report No.: 080076 GLP/GEP (Y/N): Y Published (Y/N): N
Gerke, A.	2009	XDE-208: Chronic Toxicity in Whole Sediment to Freshwater Midge *Chironomus riparius* ABC Laboratories, Inc., Columbia, Missouri, USA. DAS Report No.: 080072 GLP/GEP (Y/N): Y Published (Y/N): N

8 ANNEXES

Annex I (MoA studies for carcinogenicity)

Study 1: Ex vivo gene expression and cell proliferation analyses in rats and mice. DAR Section B.6.5.3.1.

Summary: In order to understand the basis for the Sulfoxaflor-induced rodent effects, several mode of action (MoA) investigations and studies were conducted. The initial MoA investigation was conducted on samples taken from the CD-1 mouse palatability study, where increased liver weights were first observed, and demonstrated a phenobarbital-like MoA (section B.6.5.3.1; *Geter and Kan, 2008*). Several nuclear receptors known to cause liver enlargement when stimulated by drugs and other chemicals were also investigated. These were the constitutive androstane receptor (CAR), pregnane X receptor (PXR), and peroxisome proliferator-activated receptor alpha (PPARα). In order to screen these receptors for induction following exposure to Sulfoxaflor, messenger RNA from genes associated with each of the receptors was analysed. The genes examined were *Cyp2b10* for CAR, *Cyp3a11* for PXR, and *Cyp4a10* for PPARα plus three additional CAR-related genes (*Alas1*, *Slco1b2*, and *NADPH-Cyp-reductase*). Additionally, liver tissue from mice and rats was examined using special immunohistochemical stains that label cells actively replicating. The results showed a gene expression profile in mice, and liver (hepatocellular) proliferation in both mice and rats, that was characteristic of a CAR agonist and similar to that seen following exposure to phenobarbital.

Report: Geter, D.R. and Kan, H.L. (2008). Gene Expression and Cell Proliferation Analyses in X11422208 Exposed Rats and Mice. Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan, 48674

Report No.: Study ID: 070158. US EPA MRID 47832033

Dates: Feb 2008

Guidelines: Non-guideline.

GLP: No. All experiments were done according to GLP standards and are fully reliable even though the study is not GLP compliant.

Deviations: This is acceptable as a basic though non-guideline study, it is considered supplementary to the long-term chronic / carcinogenicity studies. It reports on the analysis of samples taken from previous dietary studies.

Deficiencies: Yes, only livers from female CD1 mice were analysed with respect to specific gene expression profiling and cell proliferation. The male mouse is much more sensitive and more likely to show an effect at a specific dose level. Gene expression profiling in the female mice was conducted on liver samples from animals exposed for 3 days and not the original study period of 7 days due to palatability issues.

Executive Summary: The purpose of this study was to obtain preliminary information on the potential mode of action responsible for the liver effects observed in mice and rats from the long term studies where animals were administered dietary Sulfoxaflor. Briefly, in carcinogenicity studies in mice and rats, an increased incidence of hepatocellular tumours were identified in male rats and male and female mice. The postulated mode-of-action (MoA) for these Sulfoxaflor induced liver tumours is via a nuclear receptor-mediated mode-of-action (MoA) through the following key events: (1) constitutive androstane receptor (CAR) receptor activation and (2) increased hepatocellular proliferation, leading to (3) hepatocellular

tumours. Activation of rodent CAR and minor contributions of the pregnane X receptor (PXR) produces a cascade of alterations in gene transcription that leads to increased hepatocellular proliferation, a critical event in the development of liver tumors, similar to the established MoA for phenobarbital (PB).

This report describes (1) specific gene expression as assessed by real-time PCR in liver samples from female CD1 mice exposed to 0 and 4500ppm (345 mg/kg bw/day) dietary Sulfoxaflor for 3 days (section B.6.3.1/3a; study id 060523; *Thomas & Dryzga, 2007*); (2) cell proliferation assessed by Ki-67 immunohistochemical staining in liver tissue from 0 and 2000ppm group male and female F344 rats (155 and 170 mg/kg bw/day respectively) from the oral 28-day rat study (section B.6.3.1/2; study id 061170; *Yano et., al., 2007*) and (3) cell proliferation assessed by Ki-67 immunohistochemical staining in CD1 mouse liver tissue from 0, 3000, and 4500ppm dose groups (0, 418 and 345 mg/kg bw/day, final dose is lower due to decreased feed consumption) from the mouse palatability study (section B.6.3.1/3a; study id 060523, *Thomas & Dryzga, 2007*).

Background: A phenobarbital (PB) like mode of action (MoA) has been postulated for Sulfoxaflor induced rodent liver effects including increases in liver weight and tumour incidence. Typically, PB-induced liver enlargement and tumours involve the activation of the constitutive androstane receptor (CAR), induction of cytochrome P450 Cyp2b enzymes, particularly *Cyp2b10* in mice, hepatocellular hypertrophy, increased hepatocellular proliferation and the development of altered hepatic foci.

Results: Preliminary results indicate Sulfoxaflor induces a phenobarbital (PB)-like gene expression response consistent with CAR and PXR mediated induction of marker genes such as *Cyp2b10* (increased > 148 fold) and *Cyp3a11, Alas1, and NADPH-Cyp-reductase*. Sulfoxaflor stimulated the cholesterol synthesis-related genes, *Dhcr7* and *Sqle1*, and is not acting as a peroxisome proliferator. Sulfoxaflor increased liver hepatocyte proliferation in mice but weakly in rats: seen in the centrilobular region alone for rats and both the centrilobular and midzonal regions in mice.

Materials and Methods

Materials:

1	**Test Material:**	Sulfoxaflor
	Synonyms:	XDE-208; (N-(Methyloxido(1-(6-(trifluoromethyl)-3-pyridinyl)ethyl)-λ^4-sulfanylidene)-cyanamide); [1-(6-Trifluoromethylpyridin-3-yl)ethyl](methyl)-oxido-λ^4-sulfanylidenecyanamide; Sulfoximine; X11422208; XR-208.
	Description:	White Solid
	Lot/Batch #:	Lot # C2120-16, TSN105885
	Purity:	98.1% (w/w); as two diastereomers.
	CAS #:	946578-00-3

2	**Test Animals:**	

Species:	Mice and Rat liver samples from previous studies
Strain:	Crl:CD1(ICR) and F344/DuCrl respectively.
Other parameters:	See the individual reports (B.6.3.1/2 and B.6.3.1/3a).

Study Design:

1. Studies: Archived tissues were used for this study. For information on the animal studies that are the source of the tissues used in this investigation, see studies 060523 (B.6.3.1/3a) and 061170 (B.6.3.1/2). Briefly: in a previously conducted palatability probe study using female mice, samples of liver tissue from control, 4500 and 6000 ppm animals were collected at necropsy after 3 days of treatment, frozen in liquid nitrogen and stored at –80°C for investigation of proliferation and gene expression in liver. Given the increase in liver weight and hepatocellular hypertrophy observed with dietary Sulfoxaflor treatment, some additional research was undertaken to better characterise this response. At necropsy, livers from mice given 0, 2000,0 and 3000 ppm were preserved in formalin, whereas in the 4500 and 6000 ppm groups, half of each liver was fixed and the remaining portion was quick frozen in liquid nitrogen. In a previously conducted dietary 28-day rat study, livers from rats exposed to 0, and 2000 ppm were preserved in formalin for histological evaluation and potential cell proliferation analysis.

2. RNA sample preparation: Frozen liver samples from three control and three 4500 ppm exposed CD1 (ICR) mice were used for RNA isolation. Total RNA was extracted using the Qiagen RNeasy kit following the manufacturer's protocol. RNA quantity and quality were assessed by a NanoDrop ND-1000 spectrophotometer and Agilent 2100 Bioanalyzer, respectively. Only samples with an optical density (OD) 260/280 ratio greater than 1.8 and with clearly defined 28S and 18S bands were used for gene expression studies. Total RNA was treated with DNase enzyme to avoid DNA contamination.

3. Gene expression analysis: Gene expression studies were conducted using an Applied Biosystems 7500 real-time Polymerase Chain Reaction (PCR) system using Applied Biosystems TaqMan Gene Expression Assays. Due to the nature of the TaqMan system, dissociation curves were not required to verify the specificity of the PCR reactions.

Two housekeeping genes (constitutively active and unlikely to change with treatment) were analysed in this study: *GAPDH* and *β-Actin*. The following genes were selected to address whether Sulfoxaflor induces a phenobarbital-like gene expression response: *Cyp2b10*, *Cyp3a11*, *Slco1b2*, *Alas1*, *NADPH-Cyp-reductase*, *Dhcr7*, and *Sqle*. Five genes, primarily *Cyp2b10* but with four additional genes, were chosen to address whether Sulfoxaflor induces a phenobarbital (PB)-like gene expression response (both CAR and PXR mediated). Two genes which are also induced by PB were selected specifically to investigate the effect on blood cholesterol seen in Sulfoxaflor -treated rodents. One gene, *Cyp4a10*, was included as a marker of activated peroxisome proliferator-activated receptor alpha (PPARα) to also examine this potential pathway of action.

4. Measurement of liver cell proliferation: The livers of F344 rats and CD1 mice were analysed for the proliferation marker Ki-67 using immunohistochemical (IHC) staining to identify specific proliferating hepatocytes as determined by nuclear immunoreactivity

(*Iatropoulos and Williams, 1996*). The formalin-fixed, paraffin-embedded sample blocks were sent to Dr. Matti Kiupel (Michigan State University, Department of Pathobiology and Diagnostic Investigations, East Lansing, Michigan, USA) where the samples were sectioned and stained using standard immunohistochemical techniques (*Kiupel et al., 1999*). Slides were read at the Toxicology and Environmental Research & Consulting Unit of The Dow Chemical Company, Midland, Michigan, USA. Positive nuclei were scored as percentages based on 1000 hepatocytes in each of three hepatolobular zones per animal: centrilobular, periportal, and midzonal.

Data analysis:

1. Gene expression analysis: Gene expression was quantified using the comparative Ct method (ΔΔCt). For this method, the amount of target mRNA is expressed relative to a housekeeping gene and relative to a calibrator probe. The mRNA amounts of the 8 selected genes were calculated against the mRNA for the housekeeping gene, *β-Actin*. The mean Ct of the housekeeping gene was subtracted from the mean Ct of the target genes; the calibrator results were then subtracted from those of the control liver ($\Delta Ct_{reference}$ - ΔCt_{target} = ΔΔCt). The expression of the amount of target mRNA, normalised to an endogenous reference, and relative to a calibrator, was reported as fold change compared to control by the following formula: fold = $2^{-\Delta\Delta Ct}$. To test for significant gene expression changes, a nonparametric Wilcoxon two-tailed, two-sample test was performed using SAS 6.2 software (SAS, Cary, NC, USA) on ΔCt with alpha ≤ 0.05 considered a significant change in gene expression (*Yuan et al., 2006*).

2. Statistical analysis of Ki-67 proliferation data: Means and standard deviations were calculated for all continuous data. All parameters examined statistically were first tested for equality of variance using Bartlett's test (alpha = 0.01). If the results from Bartlett's test were significant, then the data was transformed to obtain equality of the variances. The transformations, in order, were the common log, the inverse, and the square root.

Mice: Cell proliferation data was evaluated using a 1-way ANOVA. If significant dose effects (alpha = 0.05) were determined in the 1-way ANOVA, then separate doses were compared to controls using Dunnett's test. This test corrects for experiment-wise error. The significance level is set at alpha = 0.05.

Rats: Cell proliferation data were evaluated using a 2-way ANOVA; with factors of sex and dose. The first examination was whether the sex-dose interaction was significant (alpha = 0.05); if it was, then a 1-way ANOVA was done separately for each sex. Comparisons of individual dose groups to the control group was made using Dunnett's test with the significance level set at alpha = 0.05.

Results and Discussion

A. Targeted gene expression.

Targeted gene expression analysis was carried out on female CD1 mouse liver samples from both the 0ppm control group and the 4500ppm high dose group (345 mg/kg bw/day) of a previously conducted Sulfoxaflor dietary palatability probe study. In the original study, the 4500ppm and greater dose groups were terminated after 3 days of treatment for humane reasons based on decreased feed consumption.

Specific gene expression was assessed by real-time PCR. In total, eight genes were selected

for this study. Five genes, primarily *Cyp2b10* but with four additional genes (*Cyp3a11, Alas1, NADPH-Cyp-reductase* and *Slco1b2*), were chosen to address whether Sulfoxaflor induces a phenobarbital (PB)-like gene expression response. Two genes (*Dhcr7* and *Sqle1*) which are also induced by PB were selected specifically to investigate the effect on blood cholesterol seen in Sulfoxaflor-treated rodents. One gene, *Cyp4a10*, was included as a marker of peroxisome proliferator-activated receptor alpha (PPARα) to examine this potential mode of action.

1. Housekeeping genes:

Two housekeeping genes were analysed in this study for internal standardisation of the target gene expression data: *GAPDH* and *β-Actin*. The expression values for these genes are given in table 6.5.3.1-1. Of these two housekeeping genes, *β-Actin* showed the least difference between control and treated animals and was used in all subsequent calculations to determine fold change. Gene expression results (reported as fold change) for the study are given in table 6.5.3.1-2.

Table 4.10.3.1.Study 1.1 (DAR Table 6.5.3.1-1): Gene expression of *GAPDH* and *β-Actin* housekeeping genes from control and Sulfoxaflor treated animals

Gene	Control 0ppm (Ct)	Treated 4500ppm (Ct)	Control – Treated (ΔΔCt)
GAPDH	20.845	19.626	1.219
β-Actin	21.783	21.090	0.692

GAPDH glyceraldehydes-3-phosphate dehydrogenase; The reported ΔΔCt is the fold change difference between the control and treated animals.

2. *Cyp2b10* induction:

Cyp2b10 gene induction (in mouse) is considered the prototypical gene response following phenobarbital (PB) exposure (*Ueda et al., 2002*) and activation of the nuclear receptor known as the Constitutive Androstane Receptor (CAR). In the current study, *Cyp2b10* was induced in 4500ppm Sulfoxaflor-treated mice 148.5 fold. A possible confounder in the present study was reduced feed consumption due to poor palatability in the high dose treatment groups of the original study (feed consumption was reduced by 54% compared to controls after three days of treatment). *Cyp2b10* has been reported to be induced by fasting, however mice fasted for 24 hr showed only a 2.5 fold increase in gene expression (*Ding et al., 2006*). The substantial induction of *Cyp2b10* supports the idea that Sulfoxaflor induced this gene in a PB-like manner. Any contribution to the effect by reduced feed intake was minor.

Table 4.10.3.1.Study 1.2 (DAR Table 6.5.3.1-2): Liver gene expression of selected genes from Sulfoxaflor exposed animals. Values are reported as fold change compared to control, with 95% CI.

Gene	fold-change	CI low	CI high	p value
Cyp2b10	148.51*	92.98	260.22	0.000041*
Cyp3a11	7.85*	4.45	11.48	0.000041*
Cyp4a10	-1.29	-3.86	2.84	0.7962
Slco1b2	-1.16	-1.40	1.17	0.3865
Alas1	1.51*	1.19	2.02	0.0028*
NADPH-Cyp-reductase	3.18*	2.10	4.09	0.000041*
Dhcr7	2.42*	1.88	3.06	0.000041*
Sqle1	2.05*	1.57	3.44	0.000041*

* Significant at p = 0.05.

3. Cyp3a11 induction:

Cyp3a11 gene induction is typically mediated via the PB-activated Pregnane X Receptor (PXR). This gene was reported to be induced (two to three fold) at 12 hr and three days following PB injection (*Ueda et al., 2002; Martignoni et al., 2006*) and that fasting (24 hr) produced only a small increase in expression (*Maglich et al., 2004*). In this study, *Cyp3a11* was induced 7.85 fold in the treated mice, supporting a PB-like gene expression effect.

4. *Cyp4a10* induction:

Cyp4a10 gene induction acts as a biomarker of peroxisome proliferator-activated receptor alpha (PPARα) activity, (*Patsouris, et al., 2006*) and is not induced by or associated with a PB-mode of gene expression following PB exposure, (*Ueda et al., 2002*). In the current study, *Cyp4a10* expression levels were unchanged. This suggests that Sulfoxaflor is not acting as a peroxisome proliferating compound.

5. *Slco1b2* induction:

Slco1b2 (aka. *OATP2*, *Slc21a10*) which codes for the biliary (ABC-) transporter involved in the excretion of bilirubin, bile salts, and conjugated steroids, was reported in the literature to be only slightly induced by 3.5 and 2 fold at 16hr in human hepatocytes and at five days in rats, respectively, following PB exposure (*Assenat, et al., 2004; Hagenbuch, et al., 2001*). It is thought both CAR and PXR are required for *OATP2* induction. In the current study *Slco1b2* was unchanged. The lack of *Slco1b2* induction is perhaps not surprising considering the short time-frame of PB exposure (3 days).

6. *Alas1* induction:

Alas1 codes for 5-aminolevulinic acid synthase 1 which is the first rate-limiting enzyme in the haem biosynthesis pathway providing haem for cytochrome P450s. *Alas1* was reported to be induced 4 and 8 fold at 12 and 10hr, respectively, following PB injection in mice (*Ueda et al., 2002; Fraser, et al., 2003*). The regulation of *Alas1* is thought to be CAR independent. In the current study *Alas1* was induced 1.51 fold by Sulfoxaflor, supporting its role as a PB-like

inducer.

7. *NADPH-Cyp-reductase* induction:

NADPH-Cyp-reductase (aka. *Por*) is the key enzyme that transfers electrons from NADPH to cytochrome P_{450} and was reported to be mildly induced at 12 hr following PB injection (*Ueda et al., 2002*). In the current study, *Por* was induced 3.18 fold in mice fed Sulfoxaflor.

8. *Sqle1* induction:

Sqle1 is associated with cholesterol synthesis and was reported to be induced 2.1 fold at 12 hr following PB injection (*Ueda et al., 2002*). In the current study, *Sqle1* was induced 2.05 fold.

9. *Dhcr2* induction:

Dhcr2 is also associated with cholesterol synthesis and was reported to be induced 1.6 fold at 12 hr following PB injection (*Ueda et al., 2002*). In the current study, *Dhcr2* was induced 2.42 fold. From the induced gene expression of both *Dhcr2* and *Sqle1*, it appears that Sulfoxaflor stimulates a cholesterol associated gene response in a similar manner to that observed following PB exposure.

10. *Scd1* induction:

In addition to the above-mentioned genes, an internal control gene (*Scd1*) was run to analyze and compare energy homeostasis across the experiment. The enzyme encoded by *Scd*, stearoyl-CoA desaturase 1, is involved in the synthesis of unsaturated fatty acids, as well as in the regulation of this process. *Scd1* is expressed in adipose tissue and liver. In 3T3-L1 adipocytes, *Scd1* expression is induced by insulin and suppressed by TNF, and it is activated during adipocyte differentiation (*Weiner et al., 1991; Kaestner et al., 1989*). In liver, *Scd1* expression is modulated by diet, being inhibited by fasting and induced upon re-feeding (*Ntambi, 1995*); it is down-regulated by a diet rich in polyunsaturated fatty acids (*Waters and Ntambi, 1996*). In mouse liver, *Scd1* expression and/or activity is induced by peroxisome proliferators, iron overload, and dichloroacetic acid, i.e. factors that induce hepatocellular carcinoma development or promote hepatocarcinogenesis (*Miller and Ntambi, 1996; Pigeon et al., 2001; Thai et al., 2001*). In this study, no change in *Scd1* gene expression was observed in treated mice. Animals under caloric restriction show decreased gene expression, whereas animals maintained on *ad libitum* diets show no change (*Ntambi, 1995*). The study data demonstrated that reduced feed intake in Sulfoxaflor exposed mice did not have a significant influence on gene expression. Furthermore, the unchanged *Scd1* gene expression (along with the lack of *Cyp4a10* induction), argues against a peroxisome proliferation mode of action for Sulfoxaflor.

B. Liver Cell Proliferation.

Liver proliferation results for mice and rats are shown in table 6.5.3.1-3 and table 6.5.3.1-4, respectively. Both the centrilobulor and midzonal regions in (female) mice from the 3000ppm treatment showed significant increases in cell proliferation. There were no significant alterations in proliferation in the periportal region at 3000ppm or in any region at 4500ppm. The lack of significant proliferation response at 4500 ppm was possibly due to the limited length of exposure (3 days) and lower dietary intake (due to palatability issues) when compared to the 3000 ppm group (7 days). Mice exposed to 3000 and 4500ppm Sulfoxaflor showed 53% and 40% increases in relative liver weight, respectively. The proliferation

response corresponded with pathological observations reporting greater amounts of hepatocyte mitotic figures in the 3000 ppm group than in the 4500 ppm group.

Table 4.10.3.1.Study 1.3 (DAR Table 6.5.3.1-3): Hepatocyte proliferation as measured by Ki-67 immunostaining in treated mice.

Dose	Centrilobular			Periportal			Midzonal		
	Labeled	Counted	% Labeled	Labeled	Counted	% Labeled	Labeled	Counted	% Labeled
0	3	1000	0.30	28	1000	2.80	17	1000	1.70
	5	1000	0.50	2	1000	0.20	10	1000	1.00
	9	1000	0.90	22	1000	2.20	17	1000	1.70
	6	1000	0.60	1	1000	0.10	6	1000	0.60
	2	1000	0.20	2	1000	0.20	2	1000	0.20
mean			*0.50*			*1.10*			*1.04*
3000	56	1000	5.60	6	1000	0.60	39	1000	3.90
	110	1000	11.00	17	1000	1.70	76	1000	7.60
	116	1000	11.60	18	1000	1.80	62	1000	6.20
	121	1000	12.10	8	1000	0.80	47	1000	4.70
	76	1000	7.60	3	1000	0.30	31	1000	3.10
mean			***9.58****			*1.04*			***5.10****
4500	5	1000	0.50	27	1000	2.70	15	1000	1.50
	5	1000	0.50	13	1000	1.30	5	1000	0.50
	7	1000	0.70	5	1000	0.50	5	1000	0.50
	37	1000	3.70	7	1000	0.70	16	1000	1.60
mean			*1.35*			*1.30*			*1.03*

* Significant at p = 0.05.

In rats, both sexes showed a weak but significant increase in proliferation in the centrilobulor region from the 28-day 2000 ppm treatment. There were no significant alterations in proliferation in the other regions. These findings illustrate that Sulfoxaflor exposed mice show increased proliferation in both the centrilobulor and midzonal regions following 3000ppm treatment, whereas both sexes of rat exposed to 2000ppm Sulfoxaflor showed increased proliferation only in the centrilobulor region.

In summary:

1. Sulfoxaflor shares similar gene expression with six out of seven PB-marker genes examined in mice: *Cyp2b10*, *Cyp3a11*, *Alas1*, *NADPH-Cyp-reductase*, *Dhcr7*, and *Sqle1*,

2. the lack of induction for both *Cyp4a10* and *Scd1* suggests that Sulfoxaflor was not acting as a perixisome proliferator,

3. there was no contribution of reduced feed intake to the overall gene expression,

4. Sulfoxaflor induces cholesterol synthesis genes (*Dhcr7* and *Sqle1*) to a similar extent as that observed following PB treatment,

5. increased proliferation of hepatocytes is evident in mice but less so in rats.

Table 4.10.3.1.Study 1.4 (DAR Table 6.5.3.1-4): Hepatocyte proliferation as measured by Ki-67 immunostaining in treated rats.

Dose	Centrilobular			Periportal			Midzonal		
	Labeled	Counted	% Labeled	Labeled	Counted	% Labeled	Labeled	Counted	% Labeled
0 male	4	1000	0.40	8	1000	0.80	11	1000	1.10
	6	1000	0.60	13	1000	1.30	4	1000	0.40
	7	1000	0.70	3	1000	0.30	9	1000	0.90
	3	1000	0.30	5	1000	0.50	3	1000	0.30
	6	1000	0.60	16	1000	1.60	9	1000	0.90
mean			*0.52*			*0.90*			*0.72*
0 female	6	1000	0.60	16	1000	1.60	13	1000	1.30
	2	1000	0.20	7	1000	0.70	2	1000	0.20
	2	1000	0.20	6	1000	0.60	2	1000	0.20
	3	1000	0.30	14	1000	1.40	12	1000	1.20
	9	1000	0.90	30	1000	3.00	11	1000	1.10
mean			*0.44*			*1.46*			*0.80*
2000 male	10	1000	1.00	9	1000	0.90	7	1000	0.70
	7	1000	0.70	2	1000	0.20	10	1000	1.00
	13	1000	1.30	10	1000	1.00	10	1000	1.00
	5	1000	0.50	2	1000	0.20	4	1000	0.40
mean			***0.88****			*0.58*			*0.78*
2000 female	8	1000	0.80	8	1000	0.80	8	1000	0.80
	19	1000	1.90	14	1000	1.40	7	1000	0.70
	13	1000	1.30	16	1000	1.60	5	1000	0.50
mean			***1.33****			*1.27*			*0.67*

* Statistically significant for both sexes combined versus combined control, p = 0.024.

Conclusions

These preliminary findings suggest similarities with respect to the action of phenbarbital on the rodent liver. Sulfoxaflor induces marker genes such as *Cyp2b10* (increased > 148 fold) and *Cyp3a11, Alas1, and NADPH-Cyp-reductase* consistent with CAR and PXR mediated events. Sulfoxaflor stimulated the cholesterol synthesis-related genes, *Dhcr7* and *Sqle1*, and is not acting as a peroxisome proliferator. Sulfoxaflor increased liver hepatocyte proliferation in mice but weakly in rats: seen in the centrilobular region alone for rats and both the centrilobular and midzonal regions in mice.

References

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Ding, X., Lichti, K., Kim, I., Gonzalez, F.J., Staudinger, J.L. (2006). Regulation of constitutive androstane receptor and its target genes by fasting, cAMP, hepatocyte nuclear factor alpha, and the coactivator peroxisome proliferatoractivated receptor gamma coactivator-1alpha. J. Biol. Chem., 281:26540-26551.

Fraser, D.J., Zumsteg, A., Meyer, U.A. (2003). Nuclear receptors constitutive androstane receptor and pregnane X receptor activate a drug-responsive enhancer of the murine 5-aminolevulinic acid synthase gene. J. Biol. Chem., 278:39392-39401.

Study 2: Targeted gene expression, cell proliferation and cytochrome P450 enzymatic activity in rats. DAR Section B.6.5.3.2.

A more specific experiment was conducted to rigorously examine and challenge this latter assertion

that CAR activation in a manner similar to the actions of phenobarbital was responsible for the effects of Sulfoxaflor. Fischer 344/DuCrl rats were exposed to Sulfoxaflor for either 3 or 7 days (section B.6.5.3.2; *Geter and Card, 2010*). Liver weights were increased in males and females exposed to 1500ppm Sulfoxaflor for 3 or 7 days. *Cyp2b1* gene expression, the prototypical gene response following PB exposure and CAR activation in rats, was induced over 800-fold in both male and females. Also, *Cyp2b2* and *Cyp3a3* (CAR- and PXR-related genes, respectively) were elevated as well as Cyp2b enzyme (PROD and BROD) activity levels. Furthermore, at 7 days male rats exposed to 750ppm and male and female rats at 1500ppm Sulfoxaflor showed significant hepatocellular proliferation. As in the mouse MoA study, AhR and PPARα activity was analysed and shown not to play a role in Sulfoxaflor liver effects. These results showed that the MoA most likely responsible for increased liver weight in rats was also PB-like and, as seen in the mouse studies next, males were affected to a greater extent than females.

Report: Geter, D.R., and Card, T.L. (2010). XR-208: Targeted gene expression, cell proliferation and cytochrome P450 enzymatic activity in rats. Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan, 48674.
Report No.: Study ID: 070339. DECO HET DR-0404-3134-029.
Dates: June 2010
Guidelines: Non-guideline.
GLP: No. All experiments were done according to GLP standards and are fully reliable even though the study is not GLP compliant.
Deviations: None. This is acceptable as a basic though non-guideline short term MoA study, it is considered supplementary to the long-term chronic / carcinogenicity studies.
Deficiencies: No.
Executive Summary: In previous studies targeted gene expression data in mice and hepatocellular proliferation data in both mice and rats indicated a possible phenobarbital (PB)-like mode of action (MoA) could be responsible for the liver effects related to Sulfoxaflor treatment. The purpose of this study was to determine if a PB-like MoA was responsible for the liver weight increases seen in Fischer 344 rats and to obtain information if any on dose responses of the effect. An additional aim of this study was to determine if other nuclear receptors in addition to CAR/PXR might have played a role in Sulfoxaflor-induced liver effects, namely; the aryl hydrocarbon receptor (AhR) and the peroxisome proliferator-activated receptor alpha (PPARα). Briefly, 5 male and 5 female Fischer 344/DuCrl rats per dose group were fed Sulfoxaflor in the diet at 0, 100, 750, and 1500ppm for 3 (0, 8.85, 60.3, and 99.2 mg/kg/day for males; 0, 7.83, 50.6, and 83.3 mg/kg/day for females) or 7 days (0, 8.02, 58.6, and 102 mg/kg/day for males; 0, 7.74, 53.1, and 94.4 mg/kg/day for females). The primary endpoints examined in this study were liver weight, targeted gene expression, liver enzyme analysis, and hepatocellular proliferation.

There was decreased body weight and body weight gains in males and females at the highest dose of 1500ppm after 3 and 7 days. Decreased food consumption in males and females at 750 and 1500 ppm after 3 days and in the 1500ppm group only after 7 days. There was elevated cholesterol levels in males at 750 and 1500ppm after 3 and 7 days of treatment but elevated cholesterol levels in females were only observed at 1500ppm after 7 days. At 1500ppm after 3 days the relative liver weights were increased for males only (14%), females showed a slight effect (3%); at 750 and 1500ppm after 7 days the relative liver weights were increased by 11 and 23% for males with lower increments of 6 and 18% for females, respectively. *Cyp2b1* gene expression, the prototypical gene response following PB exposure,

was induced over 800-fold in both male and female rats exposed to 1500 ppm Sulfoxaflor for 3 and 7 days. *Cyp2b2* and *Cyp3a3* (CAR- and PXR-related genes, respectively) expression levels, together with PROD and BROD enzyme activity were increased for all animals in the 750 and 1500ppm dose groups on both test days in support of a PB-like response in rodent liver. Significant hepatocellular proliferation was observed in males and females on the 2 highest doses on day 7.

Cyp1a1 gene expression and EROD enzyme activity were slightly but significantly elevated at day 3; however, EROD enzyme activity returned to control levels by day 7. In addition, gene expression of *Cyp4a22* was not elevated in this study. These results indicate no agonism or activation of the AhR or PPARα nuclear receptors. Overall, the results support the activation of CAR with contributions of the pregnane X receptor (PXR) in rodent liver when animals are exposed to Sulfoxaflor.

Materials and Methods

Materials:

1	**Test Material:**	Sulfoxaflor
	Synonyms:	XDE-208; (N-(Methyloxido(1-(6-(trifluoromethyl)-3-pyridinyl)ethyl)-λ^4-sulfanylidene)-cyanamide); [1-(6-Trifluoromethylpyridin-3-yl)ethyl](methyl)-oxido-λ^4-sulfanylidenecyanamide; Sulfoximine; X11422208; XR-208.
	Description:	White Solid
	Lot/Batch #:	Lot # E2198-17, TSN106108.
	Purity:	96.6% (w/w); as two diastereomers in 48.4 / 47.4% ratio
	Contaminants:	
	CAS #:	946578-00-3
2	**Vehicle:**	LabDiet Certified Rodent diet #5002 (PMI Nutrition International, St. Louis, Missouri, US)
	Dose	Ingested via the oral (dietary) route: Time-weighed average doses were: Males; day3: 0, 8.85, 60.3, and 99.2mg/kg body weight/day. day7: 0, 8.02, 58.6, and 102 mg/kg body weight/day. Females; day3: 0, 7.83, 50.6, and 83.3mg/kg body weight/day. day7: 0, 7.74, 53.1, and 94.4 mg/kg body weight/day.
3	**Test Animals:**	
	Species:	Rat
	Strain:	F344/DuCrl
	Age/weight at study initiation:	7-8 weeks / 0.139 – 0.202 kg (males); 0.128 – 0.145 kg (females)
	Source:	Charles River Laboratories Inc., Kingston, New York, US.
	Housing:	After assignment, animals were housed one per cage in stainless steel cages suspended above absorbent paper. Non-woven gauze was placed in the cages to provide a cushion from the flooring for rodent feet. The

	gauze and pair housing provided environmental enrichment.
Feed and Water:	LabDiet Certified Rodent Diet #5002 (PMI Nutrition International, St. Louis, Missouri, US) *ad libitum*. Municipal water *ad libitum*.
Environmental conditions:	**Temperature:** 22 ± 1 C **Humidity:** 40-70% **Air Changes:** 12-15 times/hour **Photoperiod:** 12-hour light/dark
Acclimation period:	At least one week prior to the start of the study.

Study Design:

In life dates: Test material administration for all animals began on 25th July 2007. Rats were necropsied on 28th July and 4th August 2007, respectively (days 3 and 7 of treatment).

Animal assignment and treatment groups: Before administration of test material began, animals were stratified by body weight and then randomly assigned to treatment groups. Groups of five male and five female F344/DuCrl rats were fed diets supplying 0, 100, 750, or 1500 ppm Sulfoxaflor for 3 or 7 days to obtain clinical chemistry, targeted gene expression, enzyme activity, and cell proliferation information.

Diet preparation and analysis: Diets were prepared by serially diluting a concentrated test material or feed mixture (premix) with ground feed. The concentrations of the diets were not adjusted for purity. Dose confirmation analyses of all dose levels, plus control and premix, were determined pre-exposure. The homogeneity of the low-dose and the high-dose test material-feed mixtures were determined concurrent with dose confirmation using LCMS with internal and external standards. The mean concentration for each dose level ranged from 95 to 105% of targeted concentrations, indicating acceptable concentrations of Sulfoxaflor. The homogeneity of Sulfoxaflor in diets was determined pre-exposure for the low- and high-dose diets. The relative standard deviations were 4.7% and 5.1% respectively, indicating homogeneous mixes.

Statistics: Means and standard deviations were calculated for all continuous data. All parameters were tested for equality of variance using Bartlett's test. If the results from Bartlett's test were significant at alpha = 0.01, then the data for the parameter were transformed to obtain equality of the variances.

In-life body weights were evaluated using a repeated measures (RM) analysis of variance (ANOVA), the multivariate approach, for time (the repeated factor), sex, and dose. In the repeated measures ANOVA with a pre-exposure data point, the time-dose interaction assessed the true effect of treatment. Terminal body weights, liver weights (absolute and relative), and blood cholesterol levels were evaluated using a two-way ANOVA with the factors of sex and dose. Where appropriate comparisons of individual dose groups to the control group were made with Dunnett's test (alpha = 0.05). Feed consumption data were evaluated by Bartlett's test for equality of variances. Descriptive statistics (means and standard deviations) were reported for body weight gains. Statistical outliers were identified by a sequential test (alpha = 0.02), and routinely excluded from feed consumption statistics.

Gene expression was quantified using the comparative Ct method (ΔΔCt). For this method,

the amount of target mRNA is expressed relative to a housekeeping gene and relative to a calibrator probe. The mRNA amounts of the selected genes were calculated against the mRNA for a housekeeping gene. The mean Ct of the housekeeping gene was subtracted from the mean Ct of the target genes; the calibrator results were then subtracted from those of the control liver ($\Delta Ct_{reference} - \Delta Ct_{target} = \Delta\Delta Ct$). The expression of the amount of target mRNA, normalised to an endogenous reference, and relative to a calibrator, was reported as fold change compared to control by the following formula: fold = $2^{-\Delta\Delta Ct}$. To test for significant gene expression changes, a nonparametric Wilcoxon two-tailed, two-sample test was performed using SAS 6.2 software (SAS, Cary, NC, USA) on ΔCt with alpha = 0.05 considered a significant change in gene expression.

Cell proliferation data were evaluated using a 2-way ANOVA; with factors of sex and dose. The first examination was whether the sex-dose interaction was significant (alpha = 0.05); if it was, then a 1-way ANOVA was done separately for each sex. Comparisons of individual dose groups to the control group was made using Dunnett's test with the significance level set at alpha = 0.05.

Methods:

Observations: A cage-side examination was conducted at least once a day (usually in the morning), to monitor the general health of the animals. The animals were not hand-held for these observations unless deemed necessary. Significant abnormalities that could be observed included, but were not limited to: decreased/increased activity, repetitive behaviour, vocalisation, incoordination/limping, injury, neuromuscular function (convulsion, fasciculation, tremor, or twitches), altered respiration, blue/pale skin and mucous membranes, severe eye injury (rupture), alterations in faecal consistency, and faecal/urinary quantity. In addition, all animals were observed for morbidity, mortality, and the availability of feed and water at least twice daily.

Body weight: All rats were weighed during the pre-exposure period, and on days 3 and 7 (pre-termination). Body weight gains were calculated relative to day 1.

Food consumption and compound intake: Feed consumption was determined for all animals by weighing feed containers at the start and end of a measurement cycle. The compound intake was calculated using test material concentrations in the feed, actual body weights (BW) and measured feed consumption data.

Clinical Chemistry: Limited to serum cholesterol analysis. Animals were not fasted overnight prior to blood collection. Blood samples were obtained from the orbital sinus following anesthesia with CO_2 at the scheduled necropsy. Serum cholesterol was measured using a Hitachi 912 Clinical Chemistry Analyser (Roche Diagnostics, Indianapolis, Indiana).

Targeted Gene Expression: Liver samples preserved in RNA*later*™ from all exposure groups were used for RNA isolation. Total RNA was extracted using the Qiagen RNeasy kit following the manufacturer's protocol. RNA quantity and quality were assessed by a NanoDrop ND-1000 spectrophotometer and Agilent 2100 Bioanalyzer, respectively. Only samples with an optical density (OD) 260/280 ratio greater than 1.8 and with clearly defined 28S and 18S bands were used for gene expression studies. Total RNA was treated with DNase enzyme to avoid DNA contamination. cDNA was synthesized from total RNA using TaqMan Reverse Transcription Reagents from Applied Biosystems following the manufacturer's protocol. Gene expression studies were conducted using an Applied Biosystems 7500 real-time Polymerase Chain Reaction (PCR) system using Applied

Biosystems TaqMan Gene Expression Assays.

The following genes were selected to address whether Sulfoxaflor induces a phenobarbital-like gene expression response (both CAR and PXR mediated): *Cyp2b1*, *Cyp2b2*, *Cyp3a3*, *Alas1*, *NADPH-Cyp-reductase*. Three genes, *Cyp7a1*, *Dhcr7*, and *Sqle1*, were chosen to investigate the effect on blood cholesterol seen in Sulfoxaflor -treated rodents. One gene, *Cyp4a22*, was included as a marker of activated peroxisome proliferator-activated receptor alpha (PPARα) and two others to investigate any possible AhR response (*Cyp1a1*, *Cyp1b1)*.

Measurement of liver cell proliferation: The livers of all rats were analysed for the proliferation marker Ki-67 using immunohistochemical (IHC) staining to identify specific proliferating hepatocytes as determined by nuclear immunoreactivity. The formalin-fixed, paraffin-embedded sample blocks were sent to Dr. Matti Kiupel (Michigan State University, Department of Pathobiology and Diagnostic Investigations, East Lansing, Michigan, USA) where the samples were sectioned and stained using standard immunohistochemical techniques. Slides were read at the Toxicology and Environmental Research & Consulting Unit of The Dow Chemical Company, Midland, Michigan, USA. Positive nuclei were scored as percentages based on 1000 hepatocytes in each of three hepatolobular zones per animal: centrilobular, periportal, and midzonal.

Liver Metabolic Enzyme Activities: Frozen, stored liver samples were thawed on ice, and homogenised to produce a microsomal preparation in a Tris-buffered, 20% glycerol solution containing an antioxidant (butylated hydroxyanisole), which was then frozen on dry ice, and stored at -80°C until enzyme analysis. Cyp1a enzymatic activity was measured using ethoxyresorufin (EROD), and Cyp2b activity was evaluated by benzyloxyresorufin (BROD) and pentoxyresorufin (PROD) O-dealkylase activities using a microplate fluorometric method.

Sacrifice and pathology: Non-fasted rats submitted alive for necropsy on days 3 and 7 were weighed and anesthetised by the inhalation of CO_2. Blood samples were obtained from the orbital sinus and the animals were then euthanised by decapitation. Livers were removed, weighed, and processed for analysis. The upper third of the liver left lobe was processed in RNA*later*™ for targeted gene expression analysis. The middle third of the liver left lobe, used for proliferation analysis, was trimmed and preserved in neutral, phosphate-buffered 10% formalin. The lower portion of the left lobe and the medial lobe of the liver was flash frozen and stored at -80°C for enzyme activity analysis. The remaining liver was divided, with the upper half preserved in neutral, phosphate-buffered 10% formalin and the lower half flash frozen.

Results and Discussion

Observations

Clinical signs of toxicity:

There were no clinical findings due to active substance exposure during this study. All rats survived until scheduled necropsy.

Mortality:

None.

Body weight and body weight gain

High dose rats exposed to 1500ppm Sulfoxaflor for 3 days had slightly lower body weights than controls (7% and 9% for male and female rats, respectively). This was considered to be treatment-related and was corroborated by reductions in body weight gain (60% and 101% for male and female rats, respectively) relative to concurrent controls (table 6.5.3.2-1). Reductions were also observed at the 750ppm dose level (18% and 49% for male and female rats, respectively).

For rats necropsied on day 7, male body weights from the 1500 ppm group were 7% lower than controls, females were only slightly less than controls (2%). No significant changes were observed in the 100 or 750 ppm exposure groups in either sex. Males exposed to 1500 ppm had reduced body weight gain by 44% relative to controls. Females given 750 or 1500 ppm had also reduced body weight gain (18 and 47%, respectively) relative to controls during the exposure period.

Table 4.10.3.1.Study 2.1 (DAR Table 6.5.3.2-1): Body weights and body weight gains for males and females (n = 5, all doses).

Dose (ppm)	Male				Female			
	0	**100**	**750**	**1500**	**0**	**100**	**750**	**1500**
Initial wt (g)	167.1	174.9	166.0	166.9	136.4	132.3	134.4	131.4
day 3 (g)	177.8	167.4	168.0	164.9	144.2	138.4	138.4	131.4
*% change**	*NA*	*-5.8*	*-5.5*	*-7.3*	*NA*	*-4.0*	*-4.0*	*-8.9*
day 7 (g)	198.7	209.3	195.2	184.6	147.3	150.1	147.8	144.7
*% change**	*NA*	*5.3*	*-1.8*	*-7.1*	*NA*	*1.9*	*0.3*	*-1.8*
group mean body weight gain (g)								
day 0 – 3 (g)	15.2	16.3	12.5	6.1	7.8	6.1	4.0	-0.1
*% change**	*NA*	*7.2*	*-17.8*	*-59.9*	*NA*	*-21.8*	*-48.7*	*-101*
day 0 – 7 (g)	31.6	34.5	29.2	17.8	12.7	13.2	10.4	6.7
*% change**	*NA*	*9.2*	*-7.6*	*-43.7*	*NA*	*3.9*	*-18.1*	*-47.2*

NA not applicable; *body weight (gain) % difference from controls.

Food consumption and compound intake

Feed consumption in males exposed to 750 and 1500 ppm for 3 days and 1500 ppm for 7 days was significantly lower (15 and 30% - day 3; 23% - day 7, respectively). A similar pattern of reduced food consumption was seen in females exposed to 750 and 1500 ppm for 3 days and 1500 ppm for 7 days (18 and 35%, day 3; 20% day 7, respectively). In previous studies, treatment-related differences in feed consumption were attributed to decreased palatability of Sulfoxaflor in rodent feed that resulted in lower body weight gains. Mean feed consumption data are presented in table 6.5.3.2-2.

Table 4.10.3.1.Study 2.2 (DAR Table 6.5.3.2-2): Mean Feed Consumption for males and females (n = 5, all doses).

Dose	Male	Female

(ppm)	0	100	750	1500	0	100	750	1500
day 0 – 3 (g)	15.3	14.1	13.0*	10.7*	11.2	10.6	9.2*	7.3*
day 0 – 7 (g)	15.6	15.4	14.1	12.0*	11.1	11.1	10.1	8.9*

NA not applicable; *body weight (gain) % difference from controls.

On day 3, doses of 0, 100, 750 and 1500 ppm equated to time-weighted averages of 0, 8.85, 60.3, or 99.2 mg/kg/day of the active substance Sulfoxaflor, for males and 0, 7.83, 50.6, or 83.3 mg/kg/day for females, respectively. On day 7, treatment doses equated to time-weighted averages of 0, 8.02, 58.6, or 102 mg/kg/day of the active substance Sulfoxaflor, for males and 0, 7.74, 53.1, or 94.4 mg/kg/day for females, respectively.

Clinical pathology

Clinical Chemistry:

Males exposed to 750 or 1500 ppm Sulfoxaflor and necropsied on day 3 had significantly elevated cholesterol levels of 19 and 29%, respectively. No significant changes in blood cholesterol were observed in males exposed to 100 ppm or females exposed to any concentration of Sulfoxaflor for 3 days. On day 7, males exposed to 750 and 1500 ppm and females exposed to 1500 ppm Sulfoxaflor had significantly elevated cholesterol levels of 33 and 87%, respectively, in males and 41% in females. No significant changes in blood cholesterol were observed in males exposed to 100 ppm or females exposed to 100 or 750 ppm Sulfoxaflor. Mean blood cholesterol levels are presented in table 6.5.3.2-3 below.

Table 4.10.3.1.Study 2.3 (DAR Table 6.5.3.2-3): Mean Blood Cholesterol (mg/dl) for males and females (n = 5, all doses).

Dose (ppm)	Male				Female			
	0	100	750	1500	0	100	750	1500
day 3 (±SD)	62±1	65±3	**74*±4**	**80*±3**	84±4	83±5	85±4	91±4
day 7 (±SD)	60±3	61±3	**80*±4**	**112*±8**	75±5	76±8	82±2	**106*±8**

* Data were statistically different from the control (alpha=0.05) using Dunnett's test.

Sacrifice and Pathology

Organ weights:

Both high dose male and female rats on necropsy day 3 and 7 had terminal body weights that were slightly lower (7% and 9%, and 7% and 2% respectively) than controls when analyzed across both sexes.

High dose male and female rats exposed to 1500ppm Sulfoxaflor and necropsied on day 3 had elevated relative liver weights (14% and 3%, respectively) when analysed across both sexes.

No change in relative liver weight was seen in either the 100 or 750 ppm male or female groups. At 7 days, male and female rats exposed to 750 and 1500 ppm Sulfoxaflor had significantly elevated relative liver weights of 11% and 23% for males and 6% and 18% for females, respectively, compared to controls. Absolute liver weights from male and female rats exposed to 1500 ppm Sulfoxaflor and necropsied on day 7 were significantly higher (14% and 16%, respectively) when compared to control. Mean absolute and relative liver weights are presented in table 6.5.3.2-4.

Table 4.10.3.1.Study 2.4 (DAR Table 6.5.3.2-4): Mean Liver weights for males and females (n = 5, all doses).

Dose (ppm)	Male				Female			
	0	**100**	**750**	**1500**	**0**	**100**	**750**	**1500**
Liver: (abs) day 3 (g)	7.47 ±1.05	7.24 ±0.50	7.78 ±0.94	7.84 ±0.63	5.18 ±0.24	5.09 ±0.17	4.95 ±0.32	4.87 ±0.47
Liver (rel) day 3 (g/100)	4.19 +0.21	4.33 ±0.33	4.62 ±0.24	**4.76* ±0.13**	3.59 ±0.16	3.68 ±0.10	3.58 ±0.20	**3.71* ±0.40**
Liver (abs) day 7 (g)	8.37 ±1.10	8.79 ±1.48	9.13 ±1.11	9.54 ±1.10	5.22 ±0.33	5.29 ±0.16	5.56 ±0.27	6.03 ±0.21
Liver (rel) day 7 (g/100)	4.20 ±0.25	4.18 ±0.29	**4.67* ±0.17**	**5.16* ±0.27**	3.54 ±0.11	3.53 ±0.04	**3.76* ±0.07**	4.17 ±0.08

* Data were statistically different from the control (alpha = 0.02; males and females analyzed together) using Bonferroni's test.

Microscopic pathology:

Two male high dose rats had treatment-related very slight hypertrophy of hepatocytes in the centrilobular region of the hepatic lobule after 7 days and a very slight vacuolation of hepatocytes (consistent with multifocal fatty changes). There were individual necrotic hepatocytes noted among animals, dose groups, and duration of exposure; however, the appearance was not sufficient to conclusively establish this as a treatment-related effect. The non-fasted nature of the livers and the small number of animals evaluated precluded a more detailed evaluation of possible altered cytoplasmic staining of centrilobular hepatocytes in treated rats.

Targeted gene expression, enzyme activity and hepatocyte proliferation

1. CAR and PXR associated events:

To investigate if Sulfoxaflor exposure resulted in a gene expression pattern similar to that observed with phenobarbital exposure, *Cyp2b1*, *Cyp2b2*, *Cyp3a3*, *Alas1*, and *NADPH-Cyp-reductase* expression was measured. A summary of overall liver-targeted gene expression in male and female rats exposed to 0, 100, 750, and 1500ppm Sulfoxaflor in the diet for 3 or 7

days is shown in table 6.5.3.2-5. Additionally, Cyp2b enzyme activity was evaluated by benzyloxyresorufin (BROD) and pentoxyresorufin (PROD) O-dealkylase activities (table 6.5.3.2-6).

By day 3 male and female *Cyp2b1* and *Cyp2b2* gene expression was significantly elevated at all concentrations except for female *Cyp2b1* at 100ppm. At 7 days, male *Cyp2b1* and *Cyp2b2* gene expression was significantly elevated at all test concentrations; however, female expression was significantly elevated only at 750 and 1500ppm. At 3 and 7 days, *Cyp3a3*, *Alas1*, and *NADPH-Cyp-reductase* gene expression in both sexes was significantly elevated at 750 and 1500ppm, respectively. Male and female rats exposed to 750 and 1500 ppm Sulfoxaflor for 3 or 7 days showed significantly elevated BROD and PROD enzyme activity.

2. Cholesterol metabolism:

Sulfoxaflor treatment affected serum cholesterol levels in rats, genes (*Cyp7a1*, *Dhcr7*, and *Sqle1*), involved in the metabolism and biosynthesis of cholesterol were analysed with respect to their expression profiles. *Cyp7a1* was not altered in this study. *Dhcr7* showed elevated expression only in high dose males at both 3 and 7 days (significant increases of 2.05 and 1.50-fold, respectively). *Sqle1* was only elevated in high dose males at 7 days (significant increase of 2.33-fold). No cholesterol-associated gene expression changes were observed for females in this study.

3. Other nuclear receptors:

An additional aim of this study was to examine if other nuclear receptors might have played a role in Sulfoxaflor-induced liver effects. Four nuclear receptors are primarily responsible for xenobiotic-induce liver weight increase; the aryl hydrocarbon receptor (AhR), CAR, PXR, and the peroxisome proliferator-activated receptor alpha (PPARα) (*Graham and Lake, 2008*).

Table 4.10.3.1.Study 2.5 (DAR Table 6.5.3.2-5): Targeted gene expression expressed as fold change compared to control.

3 day
Male Rat

Test Samples/Gene	*Cyp 1a1*	*Cyp 1b1*	*Cyp 2b1*	*Cyp 2b2*	*Cyp 3a3*	*Cyp 4a22*	*Cyp 7a1*	*Alas 1*	*NADPH*	*Dhcr 7*	*Sqle 1*
Control	1	1	1	1	1	1	1	1	1	1	1
100 ppm diet	1.70	1.09	9.65	2.76	1.29	-1.09	1.76	-1.01	1.09	1.04	-1.36
750 ppm diet	2.76	1.12	586.10	8.22	3.38	-1.46	1.31	1.81	1.99	1.36	1.16
1500 ppm diet	12.24	-1.14	1063.79	17.09	8.83	-1.33	1.53	2.39	3.34	2.05	1.50

Female Rat

Test Samples/Gene	*Cyp 1a1*	*Cyp 1b1*	*Cyp 2b1*	*Cyp 2b2*	*Cyp 3a3*	*Cyp 4a22*	*Cyp 7a1*	*Alas 1*	*NADPH*	*Dhcr 7*	*Sqle 1*
Control	1	1	1	1	1	1	1	1	1	1	1
100 ppm diet	1.28	1.25	3.57	2.47	1.38	-1.02	1.04	1.03	1.12	1.08	1.05
750 ppm diet	1.88	1.03	399.49	10.85	4.01	-1.40	1.54	1.68	1.69	1.06	1.09
1500 ppm diet	1.70	-1.15	1204.32	21.41	7.79	-1.24	1.10	2.64	2.78	1.04	1.20

7 day
Male Rat

Test Samples/Gene	*Cyp 1a1*	*Cyp 1b1*	*Cyp 2b1*	*Cyp 2b2*	*Cyp 3a3*	*Cyp 4a22*	*Cyp 7a1*	*Alas 1*	*NADPH*	*Dhcr 7*	*Sqle 1*
Control	1	1	1	1	1	1	1	1	1	1	1
100 ppm diet	1.08	1.27	7.19	2.92	1.16	-1.29	-1.27	1.22	1.13	1.11	1.29
750 ppm diet	3.15	-1.16	558.73	10.23	3.29	-1.85	-1.06	1.64	2.03	1.25	1.44
1500 ppm diet	12.34	1.31	848.05	21.29	9.32	-2.05	-1.05	2.94	3.23	1.50	2.33

Female Rat

Test Samples/Gene	*Cyp 1a1*	*Cyp 1b1*	*Cyp 2b1*	*Cyp 2b2*	*Cyp 3a3*	*Cyp 4a22*	*Cyp 7a1*	*Alas 1*	*NADPH*	*Dhcr 7*	*Sqle 1*
Control	1	1	1	1	1	1	1	1	1	1	1
100 ppm diet	-1.37	-1.84	2.14	1.47	-1.02	-1.16	-1.03	1.01	-1.03	-1.22	-1.14
750 ppm diet	-1.00	-1.28	315.39	6.69	3.23	-1.37	-1.04	1.75	1.55	1.11	1.14
1500 ppm diet	2.40	-1.14	855.72	11.36	6.57	-1.71	-1.05	2.03	2.45	1.27	1.21

Highlighted values were determined to be statistically different from the control (P=0.05) and ≥ 1.5-fold change criteria. All values are relative fold-change with respect to control.

Table 4.10.3.1.Study 2.6 (DAR Table 6.5.3.2-6): Liver enzyme activity of EROD (7-ethoxyresorufin-O-dealkylase), PROD (7-pentoxyresorufin-O-dealkylase), and BROD (7-benzyloxyresorufin-Odealkylase) - 3 and 7 days.

Male

3-Day	Control		100 PPM			750 PPM			1500 PPM		
Assay	**Activity**	**Std. Dev.**	**Activity**	**Std. Dev.**	**Fold**	**Activity**	**Std. Dev.**	**Fold**	**Activity**	**Std. Dev.**	**Fold**
EROD	16.08	1.53	20.34*	1.60	1.26	25.85*	1.39	1.61	24.89*	2.38	1.55
PROD	2.91	0.96	3.66	0.79	1.26	24.40*	2.70	8.38	31.66*	2.70	10.88
BROD	2.80	1.79	3.51	0.63	1.25	6.43*	0.80	2.30	4.41*	0.56	1.58

Female

3-Day	Control		100 PPM			750 PPM			1500 PPM		
Assay	**Activity**	**Std. Dev.**	**Activity**	**Std. Dev.**	**Fold**	**Activity**	**Std. Dev.**	**Fold**	**Activity**	**Std. Dev.**	**Fold**
EROD	26.64	2.82	25.29	2.40	0.95	24.90	1.13	0.93	28.81	4.76	1.08
PROD	3.73	0.25	3.33	0.34	0.89	18.34*	4.05	4.92	46.35*	6.93	12.43
BROD	3.21	2.15	3.12	0.97	0.97	16.40*	1.97	5.11	17.93*	3.81	5.59

Male

7-Day	Control		100 PPM			750 PPM			1500 PPM		
Assay	**Activity**	**Std. Dev.**	**Activity**	**Std. Dev.**	**Fold**	**Activity**	**Std. Dev.**	**Fold**	**Activity**	**Std. Dev.**	**Fold**
EROD	16.26	0.83	18.01	3.30	1.11	18.18	2.28	1.11	16.28	3.35	1.00
PROD	3.54	1.48	4.58	0.53	1.29	33.89*	2.93	9.57	35.94*	6.10	10.15
BROD	3.54	2.51	4.64	0.61	1.31	16.86*	2.00	4.76	13.20*	3.90	3.73

Female

7-Day	Control		100 PPM			750 PPM			1500 PPM		
Assay	**Activity**	**Std. Dev.**	**Activity**	**Std. Dev.**	**Fold**	**Activity**	**Std. Dev.**	**Fold**	**Activity**	**Std. Dev.**	**Fold**
EROD	31.39	2.95	30.94	1.77	0.99	31.70	1.42	1.01	26.66	1.89	0.85
PROD	4.74	3.82	5.25	0.60	1.11	61.40*	10.71	12.95	75.06*	7.01	15.84
BROD	2.57	0.75	3.07	0.65	1.19	27.56*	1.46	10.72	18.89*	2.62	7.35

*Significant by statistical evaluation ($P < 0.05$). Activity is given as pmol/min/mg protein

Aryl Hydrocarbon Receptor-Related Gene and Enzyme Activity: To address possible AhR activation by Sulfoxaflor as an alternative MoA for the liver effects seen in rats, the expression of liver *Cyp1a1* and *Cyp1b1* mRNA levels and Cyp1a enzyme activity (EROD or 7-ethoxyresorufin-O-dealkylase) was evaluated in male and female rats exposed to 0, 100, 750, or 1500ppm Sulfoxaflor in the diet for 3 or 7 days. Male rats exposed to 750 and 1500ppm Sulfoxaflor for 3 or 7 days showed significantly elevated *Cyp1a1* levels of 2.76- and 12.24-fold (3 days), and 3.15- and 12.34-fold (7 days). Females exposed to 1500ppm Sulfoxaflor for 3 days showed significantly elevated levels of *Cyp1a1* (1.70-fold), although this increase did not follow a dose-response relationship. At 7 days, elevated transcript levels were not seen at any concentration in females. *Cyp1b1* gene expression was not significantly elevated in this study in either sex at either time point (table 6.5.3.2-5).

EROD activity is a sensitive indicator of exposure to compounds eliciting receptor-mediated induction of cytochrome P_{450}-dependent monooxygenases such as Cyp1a1. Male rats exposed to Sulfoxaflor for 3 days showed mild but statistically significant increases in EROD activity at all test concentrations (1.26, 1.61, and 1.55 fold at 100, 750, and 1500 ppm, respectively). However, despite having similar increases of *Cyp1a1* mRNA at 3 and 7 days, EROD levels returned to normal in all test concentrations by 7 days, suggesting that Sulfoxaflor is unlikely to be an AhR agonist. Female EROD activity was not significantly elevated in this study (table 6.5.3.2-6).

Peroxisome Proliferation-Targeted Gene Expression: To address possible PPARα activity by Sulfoxaflor as an alternative MoA for the liver effects seen in rats, the expression of liver *Cyp4a22* mRNA levels was examined. *Cyp4a22* levels were unchanged in all 3-day samples and mildly but significantly down-regulated in 7-day males exposed to 750 or 1500 ppm (-1.85 and -2.05 fold, respectively) and 7-day females exposed to 1500 ppm Sulfoxaflor (-1.71 fold).

4. Hepatocellular Proliferation:

Liver proliferation following Sulfoxaflor exposure was examined by Ki-67 immunohistochemical staining to identify proliferating hepatocytes. Positive nuclei were scored as percentages based on 1000 hepatocytes in each of three hepatolobular zones: centrilobular, periportal, and midzonal. There were no proliferative changes in either sex at 3 days. However, at 7 days, males exposed to 750 or 1500 ppm Sulfoxaflor showed significant proliferation in both the centrilobular and midzonal regions (750 ppm - proliferation indices of 7.78 and 8.42, respectively, 1500 ppm - proliferation indices of 11.28 and 11.23, respectively; compared with the control values of 3.34 and 3.90, respectively). Females exposed to 1500 ppm Sulfoxaflor at 7 days showed significant proliferation in only the centrilobular region (proliferation index of 4.52; compared with the control value of 1.70).

Conclusions

Male and female rats exposed to dietary Sulfoxaflor showed decreased body weight gains and feed consumption along with small decrements in body weight that are not considered to be toxicologically relevant. There were no clinical signs of toxicity at any dose. In a previous short term probe and a 28-day feeding study, the observed decrease in feed consumption was attributed to decreased palatability of Sulfoxaflor enriched rodent feed and this was thought to have resulted in lower body weight gains.

Examinations of relative liver weight showed that high dose male and female rats were 14% and 3% (day 3) and 23% and 18% (day 7) higher, respectively, than controls. By day 7

absolute liver weights were increased by 14% and 18% in males and females respectively but were not found to be statistically significant. Increased liver weight, brought about by hyperplasia and hypertrophy of liver cells, is routinely observed following PB exposure in rodents (*Butler, 1978; Cunninghame et al., 1991*). Similar pathological and serological findings have also been reported in humans given PB for the treatment of epilepsy (*Whysner et al., 1996*); however no carcinogenic effects have ever been reported after long-term therapy. Although PB is not believed to cause cancer in humans, it clearly induces tumours in mice and in several rat studies. The International Agency for Research on Cancer (*IARC, 2001*) has classified PB as a Group 2B carcinogen primarily using rodent data.

Involvement of the CAR (and PXR) receptor: *Cyp2b1* gene induction is considered the prototypical gene response following PB exposure. Furthermore, *Cyp2b1* and *Cyp2b2*, are activated through the constitutive androstane receptor (CAR) with assumed cross talk with the pregnane X receptor (PXR), which stimulates the production of *Cyp3a3* in its own right (*Tien and Negishi, 2006*). CAR is a nuclear hormone receptor that detects xenobiotics and induces transcription of a number of genes regulating phase I, II, and III detoxification enzymes and transporter proteins. Both CAR and PXR belong to the NR1I nuclear receptor family (*Baes et al., 1994*). The anti-seizure drug PB is one of the best known activators of CAR (*Forman et al., 1998*). In the present study, *Cyp2b1* levels were induced over 1000-fold in both high dose male and female rats. This effect was validated in both male and female rats exposed to 750 or 1500ppm Sulfoxaflor, which showed elevated BROD and PROD enzyme activity levels at both 3 and 7 days. Additionally, *Cyp2b2* and *Cyp3a3* gene expression was significantly elevated, supporting the idea of Sulfoxaflor being an agonist for the CAR nuclear receptor resulting in the activation of CAR-related genes in a PB-like manner.

Alas1 is the first rate-limiting enzyme in the haem biosynthetic pathway that produces haem for cytochrome P_{450}. NADPH Cytochrome P_{450} reductase (also known as Por) is the key enzyme that transfers electrons from NADPH to Cytochrome P_{450}. These genes are also typically induced following PB exposure (*Ueda et al., 2002; Fraser et al., 2003*). In this study, both *Alas1* and *NADPH Cytochrome P_{450} reductase* gene expression was elevated in male and females exposed to 750 or 1500ppm Sulfoxaflor for 3 or 7 days. These data, together with *Cyp2b1*, *Cyp2b2*, *Cyp3a3* expression levels, and elevated BROD and PROD enzyme activity, support a PB-like MoA for Sulfoxaflor.

Involvement of Cholesterol: Serum cholesterol levels of male rats necropsied on day 3 and exposed to 750 or 1500 ppm Sulfoxaflor were significantly increased. On day 7, the cholesterol levels of males exposed to 750 or 1500 ppm Sulfoxaflor were significantly elevated 33 and 87%, respectively as was the cholesterol level observed from the day 7, high dose female group (41%). Increased serum cholesterol levels feature following PB exposure in both rodents and humans (*Thomas, 1984; Eiris et al., 1995*).

To examine the mechanism of increased cholesterol concentrations in the blood following Sulfoxaflor exposure, the expression of three genes with known cholesterol metabolic involvement was examined: *Cyp7a1*, *Dhcr7*, and *Sqle1*. Cyp7a1 (cholesterol 7-hydroxylase) is the principal enzyme catalysing the rate-limiting step in the intrahepatic conversion of cholesterol to bile acids (*Princen et al., 1997; Russell and Setchell, 1992*). Dhcr7 (7-dehydrocholesterol reductase) catalyses the last step in cholesterol synthesis (*Marcos et al., 2007*), whereas squalene epoxidase (Sqle1) catalyzes the first oxygenation step in sterol biosynthesis (including cholesterol) and is suggested to be one of the rate-limiting enzymes in this pathway (*Nagai et al., 1997*). Additionally, Cyp3a3 (a known PB response product) also plays a role in bile acid metabolism and elimination (*Schuetz et al., 2001*) by activation

through the PXR (*Xie et al., 2001*).

Of the examined cholesterol-associated genes, *Cyp3a3* was the most sensitive, showing elevated expression on days 3 and 7 at 750 and 1500 ppm in both male and female rats. *Dhcr7* was also elevated at 3 and 7 days, but only in 1500 ppm-exposed males. The lack of *Cyp7a1* induction in this study is not consistent with reports correlating elevated cholesterol levels to increased transcription (*Jelinek et al., 1990; Shefer et al. 1992*). Studies examining *Cyp7a1* report the gene is negatively regulated by increased levels of bile acids via the nuclear farnesoid X receptor (FXR) (*Makishima et al., 1999; Wang et al., 1999*) and positively regulated by the liver X receptor (LXR) (*Gnerre et al., 2005*). This may indicate elevated levels of bile acids or suppression of the LXR in the current study.

It has been reported that PB exposure to male Wistar rats significantly increased their serum cholesterol, increased faecal cholesterol excretion, and induced 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase activity (*Mochizuki et al., 1999*). HMG-CoA reductase is the rate-limiting enzyme of cholesterol synthesis. HMG-CoA reductase inhibitors (aka: statins) are widely used for the treatment of hypercholesterolemia and have been shown to induce CAR-associated genes (*Kobayashi et al., 2005*). Furthermore, the activation of CAR-related genes has been linked with enhanced bile acid metabolism and excretion (*Guo et al., 2003; Stanley et al., 2006*). This finding extends the cholesterol-associated genes to include those related with CAR (*Cyp2b1*, *Cyp2b2*, and to a much lesser extent *Cyp3a3*). Published studies have shown that the regulation of *Cyp2b1*, *Cyp2b2*, and HMG-CoA reductase are related and occur in parallel (*Kocarek et al., 1993*). Strain-specific responses to PB have been described in both mice and rats. In mice, an examination of four different strains (C3H, B6C3F1, C3B6F1, and C57) showed marked differences in cancer induction following PB exposure (*Lin et al., 1989*). In rats, the induction of cholesterol 7-hydroxylase and HMG-CoA reductase varied drastically among seven different strains (*Sudjana-Sugiaman et al., 1994*). Although F344 rats were not included in that study, it raises the question as to whether serum cholesterol would increase in a strain that showed decreased HMG-CoA reductase activity following PB exposure. From these findings, it can be hypothesized that PB (and possibly Sulfoxaflor) may induce hypercholesterolaemia by elevating HMG-CoA reductase activity, resulting in increased cholesterol synthesis while inhibiting the conversion of cholesterol into bile acids through suppressed cholesterol 7-hydroxylase expression.

<u>*Involvement of the Aryl hydrocarbon receptor:*</u> The aryl hydrocarbon receptor (Ah receptor or AhR) is a ligand-activated transcription factor involved in the regulation of several genes, including those for xenobiotic metabolizing enzymes such as cytochrome P450 Cyp1a1 and Cyp1b1 (*Nebert, 2000*). Ligands for the AhR include a variety of polycyclic aromatic hydrocarbons, including dioxins and related halogenated aromatic hydrocarbons, whose toxicity occurs through activation of the AhR. Male rats had a slight but significant increase in *Cyp1a1* gene expression at 750 and 1500 ppm on days 3 and 7; however, EROD activity was only increased by small amounts at all doses on day 3 and returned to normal by day 7. The lack of correspondence between *Cyp1a1* expression and EROD activity at 7 days supports the hypothesis of posttranscriptional regulation of *Cyp1a1* message in male rats exposed to Sulfoxaflor, as opposed to a receptor mediated phenomenon. Female rats aslo showed a slight increase (1.7-fold) in *Cyp1a1* at the highest dose on day 3. No treatment-related changes in EROD levels were observed in females at either 3 or 7 days.

Cyp1a1 is a non-specific indicator of AhR binding and activation (*Hu et al., 2007*). Studies report that dioxin-like toxicity requires continual presence of the AhR agonist and persistence of activation (*Wassenberg and Di Giulio, 2004; Billiard et al., 2006*). In this study,

Sulfoxaflor was administered in a manner that achieved continual presence; however, EROD activity returned to normal by day 7. This finding shows that Sulfoxaflor lacks the ability to persistently stimulate the AhR. Furthermore, an additional marker of AhR activity, *Cyp1b1* (*Badawi et al., 2000*), was not elevated in this study.

When examining the amount of cytochrome P450 present within a tissue, it is necessary to understand that each particular isozyme is present at varying levels. For example, the Cyp4a subfamily of enzymes are routinely tasked with maintaining metabolism and homeostasis of lipids, and as such are present at relatively high levels. Due to the significant amount of endogenous enzyme already present, a 10-fold increase represents a substantial amplification above the normally high amount of enzyme present. However for some P450 subfamilies such as Cyp1a and Cyp2b, levels are normally very low until induced by a specific agonist (*Baldwin et al., 2006*). Investigations using β-napthoflavone, a prototypical AhR agonist, reported *Cyp1a1* gene expression levels of > 2000 fold (*Caron et al., 2005*) and elevated EROD activity of > 1500 pmol/min/mg protein (*Sugihara et al., 2007*). The amount of *Cyp1a1* induction observed in the current study was 12-fold and the highest EROD activity was 25.85 pmol/min/mg protein. These data do not support the conclusion that Sulfoxaflor is an AhR agonist.

Involvement of the peroxisome proliferator-activated receptor (alpha) receptor: To address possible proxisome proliferation activity by Sulfoxaflor, liver *Cyp4a22* mRNA levels were examined. *Cyp4a22* is considered an indicator gene for the peroxisome proliferator-activated receptor alpha (PPARα) (*Aldridge et al., 1995*). PPARα belongs to the superfamily of nuclear receptors that can be activated by fatty acids and their metabolic derivatives. PPARα has been associated with the regulation of lipid metabolism, glucose homeostasis, cellular differentiation, cancer development, and inflammation (*Desvergne and Wahli, 1999; Duval et al., 2002; Chinetti et al., 2000*). Rats treated with the PPARα agonist clofibrate for 4 days showed elevated *Cyp4a22* of 18-fold (*Konig et al., 2007*). Interestingly, *Konig et al.,* (2007) reported that elevated *Cyp4a22* levels following clofibrate exposure correlated with decreased levels of serum cholesterol. In the current study, *Cyp4a22* levels were unchanged in all day 3 samples and significantly down-regulated in day 7 males exposed to 750 or 1500ppm and 7 day females exposed to 1500ppm Sulfoxaflor. In mice exposed to PB, gene expression of the homologue to *Cyp4a22* was similarly decreased but only by about 1.6 fold (*Ueda et al., 2002*). These data show that Sulfoxaflor potentially suppressed *Cyp4a22* gene expression, inconsistent with PPARα activation and is possibly linked to increased cholesterol synthesis.

Hepatocellular proliferation: Liver proliferation following Sulfoxaflor exposure was examined by Ki-67 immunohistochemical staining to identify proliferating hepatocytes. There were no proliferative changes in either sex after 3 days of treatment. However, after 7 days of treatment, males exposed to 750 and 1500 ppm showed significant proliferation in both the centrilobular and midzonal regions. High dose females exposed for 7 days showed significant proliferation in only the centrilobular region. The observed proliferation does not appear to be induced by cytolethality, but rather through a mitogenic mechanism resulting in increased cell number and organ size. Moreover, nuclear receptor ligands can exert a direct hyperplastic effect on the liver by stimulating hepatocyte proliferation (*Columbano and Ledda-Columbano, 2003*). Studies examining liver proliferation in PB-exposed rats have shown significant increases by 7 days (*Peraino et al., 1971*) focused mainly in the centrilobular region (*Kolaja et al., 1996*). These data demonstrate that the hepatocellular proliferation induced following Sulfoxaflor exposure is similar to that observed following PB exposure.

Based upon these results, increased liver weight in rats administered dietary Sulfoxaflor was similar to the action of phenobarbital, as evidenced by the CAR and PXR-related molecular, enzymatic, and proliferative responses.

IV. References

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Study 3: Mode of Action Study Investigating Liver Weight Effects in Crl:CD-1(ICR) Mice. DAR Section B.6.5.3.3.

The MoA of sulfoxaflor-induced liver effects in CD-1 mice was also further examined in a similar manner to the one conducted in Fischer 344/DuCrl rats. Male and female CD-1 mice were exposed to sulfoxaflor in the diet for 7 days along with an examination of liver samples from the 28 and 90-day sulfoxaflor mouse studies (section B.6.5.3.3; *Geter et al., 2010*). Hepatocyte hypertrophy was observed in high dose males exposed to 750ppm and females exposed to 1000 and 1500ppm sulfoxaflor. Targeted gene expression, as in the earlier mouse study (section B.6.5.3.1; *Geter and Kan, 2008*), showed a profile characteristic of a compound primarily stimulating CAR. Of note was the significant induction of *Cyp2b10*, considered the primary CAR-response gene following PB exposure. Increased transcription levels of *Cyp2b10* generally lead to increased levels of Cyp2b10 protein and therefore, measuring 7-pentoxyresorufin-O-dealkylase (PROD) and 7-benzyloxyresorufin-O-dealkylase (BROD) enzyme activities provides a quantitative measure of the metabolic activity of the Cyp2b10 protein. Both PROD and BROD liver activities were significantly elevated in both male and female mice at all time-points. Hepatocellular proliferation at 7 days in male mice exposed to 750ppm, and females exposed to 750 and 1500ppm was statistically elevated. Induction of the aryl hydrocarbon receptor (AhR) was also examined as activation of this receptor can also lead to increased liver weight. Neither AhR nor PPARα were concluded to be involved in the liver alterations induced by sulfoxaflor. Based upon these results, the sulfoxaflor-induced liver specific effects in mice were consistent with CAR activation

resulting in a PB-like MoA and that male mice were more sensitive to the effects of sulfoxaflor then female mice.

Report: Geter, D. R., Murray, J. A., L.V.T., Kan, H. L., LeBaron, M. J. and Thomas, J. (2010). XDE-208: Mode of Action Study Investigating Liver Weight Effects in Crl:CD-1(ICR) Mice. Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan, 48674.

Report No.: Study ID: 080246. DECO HET DR-0404-3134-041.

Dates: March 2008

Guidelines: Non-guideline.

GLP: No. All experiments were done according to GLP standards.

Deviations: None. This is acceptable as a basic though non-guideline short term MoA study, it is considered supplementary to the long-term chronic / carcinogenicity studies.

Deficiencies: No.

Executive Summary: In previous studies limited targeted gene expression data in mice, a more comprehensive study of targeted gene expression in rats and hepatocellular proliferation data in both mice and rats indicated a possible phenobarbital (PB)-like mode of action (MoA) could be responsible for the liver effects related to sulfoxaflor treatment. The purpose of this study was to investigate in further detail if a PB-like MoA was responsible for liver weight increases seen in CD-1 mice following sulfoxaflor exposure and to obtain information on a possible dose response of the effect or if the effects follow on from a threshold limit. In concert with the rat study described previously (section B6.5.3.2); an additional aim of this study was to determine if other nuclear receptors in addition to CAR/PXR might have played a role in sulfoxaflor-induced liver effects, namely; the aryl hydrocarbon receptor (AhR) and the peroxisome proliferator-activated receptor alpha (PPARα).

Briefly, 5 male and 5 female CD-1 mice per dose group were fed sulfoxaflor in the diet at either 0, 500, and 750ppm for males (0, 89, and 128mg/kg bw/day), or 0, 1000, and 1500ppm for females (0, 211, and 323mg/kg bw/day) for a total of 7 days. The primary endpoints examined in this study were liver weight, targeted gene expression, liver enzyme analysis, and hepatocellular proliferation. In addition, archived liver samples from previously conducted 28 and 90-day sulfoxaflor mouse studies were analysed for targeted gene expression, liver enzyme activity, and hepatocellular proliferation (Ki-67).

Liver weights increased with treatment dose of sulfoxaflor. High dose males (750ppm) had an absolute liver weight increase of 14% (17% in relative liver weight) compared with controls. The effect was greater in females on higher dose treatments, mean group liver weights increased by 43% and 47% (38% and 43% for relative liver weight) in animals exposed to dietary levels of 1000ppm and 1500ppm respectively. These liver weight increases correlated with treatment-related observations of centrilobular and midzonal hepatocyte hypertrophy with very slightly increased cytoplasmic eosinophilia. There was also evidence of lipid changes in the hepatocytes of high dose males, and increased numbers of mitotic hepatocytes and individual cell necrosis at doses ≥ 500ppm in both sexes.

An elevation in *Cyp2b10* levels was characteristic of all animals exposed to sulfoxaflor and

liver samples from the 28 and 90-day studies. Males generally had a higher response than females, i.e. they were more sensitive, even though their systemic exposures were lower. These results for *Cyp2b10* mRNA concurred with increased PROD and BROD liver enzyme activities in all animals on all doses. Similarly, *Cyp3a11* levels were also elevated in high dose males and all female dose groups. Hepatocyte proliferation was also evident from results using the BrdU and Ki-67 immunohistochemical techniques. Ki-67 analysis of hepatocellular proliferation was less sensitive then BrdU, in contrast to the BrdU results, increased proliferation was not evident at any dose or zone by Ki-67 analysis. Ki-67 analysis of hepatocellular proliferation in the 28 and 90-day studies showed no induction at either time point in male or female mice.

AhR-related EROD liver enzyme activity was slightly elevated in this study at all time-points in both male and female mice; however, the degree of induction was mild (none greater than 2.3-fold) and may be associated with the large induction of Cyp2b enzyme. *Cyp4a10*, a PPARα related gene, was not significantly altered in this study.

Overall, the results support the idea of a PB-like response by the liver when animals are exposed to sulfoxaflor.

Materials and Methods

Materials:

1	**Test Material:**	Sulfoxaflor
	Synonyms:	XDE-208; (N-(Methyloxido(1-(6-(trifluoromethyl)-3-pyridinyl)ethyl)-λ^4-sulfanylidene)-cyanamide); [1-(6-Trifluoromethylpyridin-3-yl)ethyl](methyl)-oxido-λ^4-sulfanylidenecyanamide; Sulfoximine; X11422208; XR-208.
	Description:	White Solid
	Lot/Batch #:	Lot # E2162-34, TSN003725-0001.
	Purity:	95.6% (w/w); as two diastereomers in 48.4 / 47.4% ratio
	Contaminants:	
	CAS #:	946578-00-3
2	**Vehicle:**	LabDiet Certified Rodent diet #5002 (PMI Nutrition International, St. Louis, Missouri, US)
	Dose	Ingested via the oral (dietary) route: Time-weighed average doses were: Males; day7: 0, 89, 128 mg/kg body weight/day. Females; day7: 0, 211, 323 mg/kg body weight/day.
3	**Test Animals:**	
	Species:	Mouse
	Strain:	Crl:CD1(ICR)
	Age/weight at study initiation:	6 weeks / 0.029 – 0.029 kg (males); 0.022 – 0.022 kg (females)
	Source:	Charles River Laboratories Inc., Kingston, New York, US.
	Housing:	After assignment, animals were housed one per cage in stainless steel cages suspended above absorbent paper. Non-woven gauze was placed in the cages to provide a cushion

	from the flooring for rodent feet. The gauze and pair housing provided environmental enrichment.
Feed and Water:	LabDiet Certified Rodent Diet #5002 (PMI Nutrition International, St. Louis, Missouri, US) *ad libitum*. Municipal water *ad libitum*.
Environmental conditions:	**Temperature:** 22 ± 1 C **Humidity:** 40-70% **Air Changes:** 12-15 times/hour **Photoperiod:** 12-hour light/dark
Acclimation period:	At least one week prior to the start of the study.

Study Design:

In life dates: Test material administration for all animals began on 6th March 2008. Mice were necropsied on 13th March 2008, (day 7 of treatment).

Animal assignment and treatment groups: Before administration of test material began, animals were stratified by body weight and then randomly assigned to treatment groups. Groups of five male and five female CD-1 mice were fed diets supplying 0, 500, and 750ppm sulfoxaflor (males) or in the case of females 0, 1000, and 1500ppm sulfoxaflor for 7 days to obtain clinical chemistry, targeted gene expression, enzyme activity, and cell proliferation information.

Diet preparation and analysis: Diets were prepared by serially diluting a concentrated test material or feed mixture (premix) with ground feed. The concentrations of the diets were not adjusted for purity. Dose confirmation analyses of all dose levels, plus control and premix, were determined pre-exposure. The homogeneity of the low-dose and the high-dose test material-feed mixtures were determined concurrent with dose confirmation using LCMS with internal and external standards. The mean concentration for each dose level ranged from 95 to 108% of targeted concentrations, indicating acceptable concentrations of sulfoxaflor. The homogeneity of sulfoxaflor in diets was determined pre-exposure for the low- and high-dose diets. The relative standard deviations were 4.8% and 6.4% respectively, indicating homogeneous mixes.

Statistics: Means and standard deviations were calculated for all continuous data. All parameters were tested for equality of variance using Bartlett's test. If the results from Bartlett's test were significant at alpha = 0.01, then the data for the parameter were transformed to obtain equality of the variances.

In life body weights, terminal body weight, liver weight, relative liver weight, cholesterol, ALT, AST, enzyme activity, BrdU, and Ki67 proliferation were evaluated using a one-way ANOVA with the factor of dose. Where appropriate comparisons of individual dose groups to the control group were made with Dunnett's test (alpha = 0.05). Feed consumption data were evaluated by Bartlett's test for equality of variances. Descriptive statistics (means and standard deviations) were reported for body weight gains. Statistical outliers were identified by a sequential test (alpha = 0.02), and routinely excluded from feed consumption statistics.

Gene expression was quantified using the comparative Ct method (ΔΔCt). For this method, the amount of target mRNA is expressed relative to a housekeeping gene and relative to a calibrator probe. The mRNA amounts of the selected genes were calculated against the mRNA for a housekeeping gene. The mean Ct of the housekeeping gene was subtracted from

the mean Ct of the target genes; the calibrator results were then subtracted from those of the control liver ($\Delta Ct_{reference}$ - ΔCt_{target} = $\Delta\Delta Ct$). The expression of the amount of target mRNA, normalised to an endogenous reference, and relative to a calibrator, was reported as fold change compared to control by the following formula: fold = $2^{-\Delta\Delta Ct}$. To test for significant gene expression changes, a nonparametric Wilcoxon two-tailed, two-sample test was performed using SAS 6.2 software (SAS, Cary, NC, USA) on ΔCt with alpha 0.05 considered a significant change in gene expression.

Methods:

Observations: A cage-side examination was conducted at least once a day (usually in the morning), to monitor the general health of the animals. The animals were not hand-held for these observations unless deemed necessary. Significant abnormalities that could be observed included, but were not limited to: decreased/increased activity, repetitive behaviour, vocalisation, incoordination/limping, injury, neuromuscular function (convulsion, fasciculation, tremor, or twitches), altered respiration, blue/pale skin and mucous membranes, severe eye injury (rupture), alterations in faecal consistency, and faecal/urinary quantity. In addition, all animals were observed for morbidity, mortality, and the availability of feed and water at least twice daily.

Body weight: All mice were weighed during the pre-exposure period (before osmotic pump implantation) and then on day 7. Body weight gains were calculated relative to day 1.

Food consumption and compound intake: Feed consumption was determined for all animals by weighing feed containers at the start and end of a measurement cycle. The compound intake was calculated using test material concentrations in the feed, actual body weights (BW) and measured feed consumption data.

Clinical Chemistry: Animals were not fasted overnight prior to blood collection. Blood samples were obtained from the orbital sinus following anesthesia with Isoflurane/O_2 at the scheduled necropsy. Serum parameters (cholesterol, CHOL; alanine aminotransferase, ALT; aspartate aminotransferase, ASP and triglycerides, TG) were measured using a Hitachi 912 Clinical Chemistry Analyser (Roche Diagnostics, Indianapolis, Indiana).

Targeted Gene Expression: Liver samples preserved in RNA*later*™ from all exposure groups were used for RNA isolation. Total RNA was extracted using the Qiagen RNeasy kit following the manufacturer's protocol. RNA quantity and quality were assessed by a NanoDrop ND-1000 spectrophotometer and Agilent 2100 Bioanalyzer, respectively. Only samples with an optical density (OD) 260/280 ratio greater than 1.8 and with clearly defined 28S and 18S bands were used for gene expression studies. Total RNA was treated with DNase enzyme to avoid DNA contamination. cDNA was synthesized from total RNA using TaqMan Reverse Transcription Reagents from Applied Biosystems following the manufacturer's protocol. Gene expression studies were conducted using an Applied Biosystems 7500 real-time Polymerase Chain Reaction (PCR) system using Applied Biosystems TaqMan Gene Expression Assays.

The following genes were selected to address whether sulfoxaflor induces a phenobarbital-like gene expression response (both CAR and PXR mediated): *Cyp2b10*, *Cyp3a11*, and *Cyp4a10* was included as a marker of activated peroxisome proliferator-activated receptor alpha (PPARα).

Measurement of liver cell proliferation: Incorporation of 5-bromo-2'-deoxyuridine (BrdU;

a structural analog of thymidine), into nuclear DNA was used as a surrogate marker of cell proliferation. One day prior to exposure to the sulfoxaflor treated diets, all study animals were implanted with mini-osmotic pumps (Model 2001; Alzet Corporation, Palo Alto, California). Mice were continuously infused with BrdU via the implanted osmotic pumps filled with a 20mg/ml solution of BrdU in phosphate buffered saline (pH 7.6), at a delivery rate of 1µl/hour. Levels of hepatocyte S-phase DNA synthesis were determined using BrdU immunohistochemistry. Using light microscopy, a labeling index was calculated and positive nuclei were scored as percentages based on 1000 hepatocytes in each of three hepatolobular zones: centrilobular, midzonal, and periportal regions.

In addition, the livers of all mice were analysed for the proliferation marker Ki-67 using immunohistochemical (IHC) staining to identify specific proliferating hepatocytes as determined by nuclear immunoreactivity. The formalin-fixed, paraffin-embedded sample blocks were sent to Dr. Matti Kiupel (Michigan State University, Department of Pathobiology and Diagnostic Investigations, East Lansing, Michigan, USA) where the samples were sectioned and stained using standard immunohistochemical techniques. Slides were read at the Toxicology and Environmental Research & Consulting Unit of The Dow Chemical Company, Midland, Michigan, USA. Positive nuclei were scored as percentages based on 1000 hepatocytes in each of three hepatolobular zones per animal: centrilobular, periportal, and midzonal.

Liver Metabolic Enzyme Activities: Frozen, stored liver samples were thawed on ice, and homogenised to produce a microsomal preparation in a Tris-buffered, 20% glycerol solution containing an antioxidant (butylated hydroxyanisole), which was then frozen on dry ice, and stored at -80°C until enzyme analysis. Cyp1a enzymatic activity was measured using ethoxyresorufin (EROD), and Cyp2b activity was evaluated by benzyloxyresorufin (BROD) and pentoxyresorufin (PROD) O-dealkylase activities using a microplate fluorometric method.

Sacrifice and pathology: Non-fasted mice submitted alive for necropsy on day 7 were weighed and anesthetised by the inhalation of Isoflurane/O_2. Blood samples were obtained from the orbital sinus and the animals were then euthanised by decapitation. Livers were removed, weighed, and processed for analysis. The upper third of the liver left lobe was processed in RNA*later*™ for targeted gene expression analysis. The middle third of the liver left lobe, used for proliferation analysis, was trimmed and preserved in neutral, phosphate-buffered 10% formalin. The lower portion of the left lobe and the medial lobe of the liver was flash frozen and stored at -80°C for enzyme activity analysis. The remaining liver was divided, with the upper half preserved in neutral, phosphate-buffered 10% formalin and the lower half flash frozen. Archived liver from the 28 and 90-day dietary mouse studies were taken from both lateral and medial lobes (left and right) and processed in a similar manner to that already described above.

Results and Discussion

Observations

Clinical signs of toxicity:

There were no clinical findings due to active substance exposure during this study. All mice survived until scheduled necropsy.

Mortality:

None.

Body weight and body weight gain

There were no statistically identified differences in body weights of male or female mice at any dose level compared to control animals throughout the study.

Food consumption and compound intake

Feed consumption values for females given either 1000 or 1500ppm were lower than controls and identified as statistically significant and treatment-related. Feed consumption values for females given 1000 or 1500 ppm were 12% and 10% lower than controls on day 7. Feed consumption for males at all dose levels was comparable to controls throughout the study. During this study, doses of 0, 500 and 750ppm equated to time-weighted averages of 0, 89, or 128 mg/kg/day of the active substance sulfoxaflor, for males. Doses of 0, 1000 and 1500ppm equated to time-weighted averages of and 0, 211, or 323 mg/kg/day for females.

Clinical pathology

Clinical Chemistry:

The clinical chemistry parameters measured were triglycerides (TG), cholesterol (CHOL), alanine aminotransferase (ALT), and aspartate aminotransferase (AST). Triglycerides were slightly elevated in high dose females (1500ppm) although the difference from control was not statistically significant. (See table 6.5.3.3-1) This was considered treatment-related because increases in triglycerides have been noted in females given 3500ppm for 28 days or 1500ppm or greater for 90 days. Curiously, in the 28-day study, triglycerides were not elevated in females at 1500ppm, the reason for this discrepancy is not clear. There were no other treatment-related changes in either sex for these clinical chemistry parameters.

Table 4.10.3.1.Study 3.1 (DAR Table 6.5.3.3-1): Summary of Clinical Chemistry data for males and females (n = 5, all doses).

Dose (ppm)	Male			Female		
	0	500	750	0	1000	1500
TG (±SD)	101±19	93±14	88±18	78±18	103±35	131±36
CHOL (±SD)	119±13	118±19	112±7	82±18	96±15	84±8
ALT (±SD)	105±79	73±54	101±53	102±56	106±81	120±82
AST (±SD)	110±14	125±18	138±45	188±66	200±67	193±37

Sacrifice and Pathology

Organ weights:

There were no differences in final body weights of male or female mice when compared to their respective controls. Liver weights were increased in high dose males (14%) relative to controls and females from the 1000 or 1500 ppm had increases of 43% and 47% respectively. Relative liver weights were also increased; up to 17% in high dose males, while females from the 1000 or 1500 ppm had increases of 39% and 43% respectively.

Microscopic pathology:

High dose males and females receiving 1000 or 1500ppm had treatment-related hypertrophy of hepatocytes involving the centrilobular and midzonal regions of the hepatic lobule. Accompanying the hypertrophy, affected hepatocytes had a very slightly increased eosinophilia (altered tinctorial properties consistent with enzyme induction and/or smooth endoplasmic reticulum increase). There were very slight treatment-related increases in the number of mitotic hepatocytes in some males given 500 or 750ppm and in the majority of females given 1000 or 1500ppm. Minimal focal, or very slight multifocal individual cell necrosis of hepatocytes occurred in some males given 500 or 750ppm and in females given 1000 or 1500ppm characterised by the presence of 1 or 2 necrotic hepatocytes (minimal focal) or 5-6 scattered necrotic hepatocytes (very slight multifocal) in the entire liver section. These isolated necrotic hepatocytes were usually surrounded or infiltrated with one or more neutrophils.

Table 4.10.3.1.Study 3.2 (DAR Table 6.5.3.3-2): Incidence (number of animals) of selected histopathological liver effects (n = 5 livers examined, all doses)

		Males			Females		
Dose (ppm)		**0**	**500**	**750**	**0**	**1000**	**1500**
Hypertrophy; with altered tinctorial properties; hepatocyte; centrilobular / midzonal.-	very slight	0	0	5	0	4	5
Necrosis; individual cell;							
hepatocyte;	minimal	0	1	2	0	1	1
focal:	very	0	1	1	0	0	0
multifocal:	slight						
Mitotic alteration; increased;	very	0	2	3	1	3	4
hepatocyte, multifocal:	slight	0	0	0	1	1	1
	slight						

Fresh frozen sections of the liver from the first 3 necropsied males (control and 750ppm groups) and the first 3 necropsied females (control and 1500ppm groups) from the current study were stained with Oil Red O for evaluation of cytoplasmic lipid in the hepatocytes. Minimal changes were observed: 2 out of the 3 control males had very slight lipid staining, while 2 of 3 high dose males had a slight increase in lipid staining. There was no treatment related difference in the amounts of cytoplasmic lipid in hepatocytes of high dose females relative to the controls.

In addition to the above, Oil Red O staining was conducted on selected male mouse liver sections obtained from a previously conducted 90-day dietary study with sulfoxaflor. Frozen sections of liver (fixed and preserved in formalin) from three selected males from control

group and 750ppm group were stained with Oil Red O and also demonstrated a slight increase in cytoplasmic lipid in the hepatocytes from the high dose group.

Targeted gene expression, enzyme activity and hepatocyte proliferation

1. CAR and PXR associated events:

To investigate if sulfoxaflor exposure resulted in a gene expression pattern similar to that observed with phenobarbital exposure, *Cyp2b10* and *Cyp3a11* were measured. Additionally, Cyp2b enzyme activity was evaluated by benzyloxyresorufin (BROD) and pentoxyresorufin (PROD) O-dealkylase activities. *Cyp2b10* gene induction, considered to be the prototypical gene response following PB exposure through activation of the constitutive active/androstane nuclear receptor (CAR), was induced 42.1 and 54.8-fold in the 500 and 750ppm males, respectively, while females given 1000 and 1500ppm were induced 20.0 and 30.8-fold, respectively, relative to controls (table 6.5.3.3-3). In examining *Cyp2b10* gene expression in males from the previous 28 and 90 day studies, the expression was increased 61.7-fold (300 ppm) in the 28 day study and 56.5-fold (750 ppm) in the 90 day study (relative to controls). Females exposed to 1500ppm in these past studies also showed elevated *Cyp2b10* expression at 28 and 90 days (93.9 and 53.9-fold, respectively). *Cyp3a11*, which is also associated with PB-like gene expression through the pregnane X nuclear receptor (PXR), was significantly elevated in the high dose males (and in the reviously performed 90 day study), while female mice were significantly elevated at all doses (also true in previous 28 and 90 day studies).

Table 4.10.3.1.Study 3.3 (DAR Table 6.5.3.3-3): Targeted gene expression expressed as fold change compared to control.

		Male			Female		
study	**dose (ppm)**	***Cyp2b10***	***Cyp3a11***	***Cyp4a10***	***Cyp2b10***	***Cyp3a11***	***Cyp4a10***
7 day	0	1.0	1.0	1.0	1.0	1.0	1.0
	500	42.1*	1.6	1.2			
	750	54.8*	2.7*	1.0			
	1000				20.0*	4.0*	-5.6
	1500				30.8*	6.6*	-3.3
28 day	300	61.7*	1.5	-1.6			
	1500				93.9*	5.6*	-1.8
90 day	750	56.5*	2.8*	-3.6			
	1500				53.9*	3.4*	-2.3

* Data were statistically different from the control (alpha = 0.05) and fold-change > 1.5

Both PROD and BROD liver enzyme activities, which are associated with CAR activation and give a measure of Cyp2b enzyme induction, were likewise significantly elevated in this study (and in the 28 and 90 day studies) in both male and female mice (range of 2.56 to 9.49-fold, table 6.5.3.3-4).

Table 4.10.3.1.Study 3.4 (DAR Table 6.5.3.3-4): Liver enzyme activity of EROD, PROD and BROD.

		Male			Female		
study	**dose (ppm)**	**EROD**	**PROD**	**BROD**	**EROD**	**PROD**	**BROD**
7 day	0	20.3	1.2	2.7	10.5	2.8	2.2
	500	30.1*	5.1*	18.0*			
	750	33.3*	5.4*	19.7*			
	1000				23.3*	13.9*	7.7*
	1500				23.0*	13.8*	8.6*

28 day	0	16.8	1.8	2.3	12.7	2.7	3.3
	300	23.8*	8.8*	21.4*			
	1500				22.9*	6.9*	14.9*
90 day	0	20.1	4.0	5.2	16.4	5.1	6.1
	750	24.8*	15.5*	22.0*			
	1500				24.5*	20.0*	24.4*

* Data were statistically different from the control (alpha = 0.05)

2. Aryl Hydrocarbon Receptor Related Enzyme Activity (EROD):

The activity of liver Cyp1a enzyme activity (EROD, 7-ethoxyresorufin-O-dealkylase) which is associated with AhR activity, was significantly elevated in this study in both male and female mice at all time-points (table 6.5.3.3-4).

3. Peroxisome Proliferation Targeted Gene Expression (*Cyp4a10*):

Cyp4a10, a PPARα-related gene, was not significantly altered in this study in either sex at any time point (table 6.5.3.3-3).

4. Hepatocellular Proliferation:

Liver proliferation following sulfoxaflor exposure was examined by BrdU analysis and Ki-67 immunohistochemical staining to identify proliferating hepatocytes. Positive nuclei were scored as percentages based on 1000 hepatocytes in each of three hepatolobular zones: centrilobular, periportal, and midzonal.

BrdU analysis showed that males exposed to 750ppm sulfoxaflor had significant proliferation in both the centrilobular and midzonal regions, but at 500ppm only the centrilobular region showed significant proliferation. Females exposed to 1000 and 1500ppm showed significant proliferation as measured by BrdU in all three zones of the liver. Males exposed to 500 and 750ppm sulfoxaflor showed significant proliferation in the centrilobular region, however, induction was not observed in the midzonal region of the 750ppm-treated males, which was positive by BrdU. In females, there were no statistically identified increases in proliferation at any dose or zone by Ki-67 immunohistochemistry, whereas both doses and all zones were positive by BrdU analysis.

Table 4.10.3.1.Study 3.5 (DAR Table 6.5.3.3-5): Hepatocyte proliferation as measured by BrdU in treated mice (n = 5 animals per dose per sex).

Dose	**Centrilobular**		**Periportal**		**Midzonal**	
(ppm)	male	female	male	female	male	female
0	2.30	8.90	2.64	8.20	1.46	8.40
500	**6.16***		3.72		4.92	
750	**7.33***		3.83		**5.63***	
1000		**36.70***		**22.10***		**30.04***
1500		**43.32***		**31.02***		**39.12***

* Significant at alpha = 0.05 (Dunnett's test); score: mean % positive hepatocytes based on 1000 cells/zone/animal.

Table 4.10.3.1.Study 3.6 (DAR Table 6.5.3.3-6): Hepatocyte proliferation as measured by Ki-67 immunostaining in treated mice (n = 5 animals per dose per sex).

Dose	Centrilobular		Periportal		Midzonal	
(ppm)	male	female	male	female	male	female
0	0.54	0.83	0.70	0.40	0.54	0.87
500	**1.10***		0.54		0.98	
750	**1.10***		0.55		0.78	
1000		2.36		0.80		1.98
1500		1.80		0.64		2.08

* Significant at alpha = 0.05 (Dunnett's test); score: mean % positive hepatocytes based on 1000 cells/zone/animal.

Proliferation analysis of the 28 and 90-day liver samples was also performed and statistical analysis did not identify any treatment related differences in proliferation at the doses tested, as measured by Ki-67.

Conclusions

Male and female mice exposed to dietary sulfoxaflor showed no effect on body weight gains, a slight decrease in feed consumption was noted for females and is attributed to palatability issues, and there were no significant changes in body weight of either sex at any dose level. There were no clinical signs of toxicity at any dose.

High dose males had a statistically identified increase (17%) in relative liver weights compared to controls (absolute liver weight was increased by 14%). Furthermore, females exposed to 1000 and 1500ppm had statistically identified increases in relative (38 and 43%, respectively) and absolute (43 and 47%, respectively) liver weights compared to controls. These liver weight increases correlated with treatment-related observations of centriloboular/midzonal hepatocyte hypertrophy with very slightly increased cytoplasmic eosinophilia. The latter observation is consistent with sulfoxaflor mediated induction of metabolising enzymes and/or increase in the amount of smooth endoplasmic reticulum. Hepatocyte hyperplasia was also evident in males given 500 or 750ppm and in females exposed to 1000ppm or 1500ppm due to very slight increases in the numbers of mitotic hepatocytes. High dose males (750ppm) at either 7 or 90 days showed a very slight or slight increase in lipid within the hepatocytes compared to controls. Minimal or very slight individual hepatocyte necrosis was observed in some males given 500ppm or 750 ppm and in some females given 1000ppm or 1500 ppm. The exact mechanism of sulfoxaflor induced hepatocyte proliferation and other hepatocyte effects is unclear. However, due to the similarities in PB-like gene expression profiles and related endpoints (such as increased liver weight), it is thought that the hepatocyte effects are mediated through CAR/PXR nuclear receptor activation.

Involvement of the CAR (and PXR) receptor: *Cyp2b10* gene induction is considered the

prototypical gene response following PB exposure in mice. Furthermore, *Cyp3a11* which is mainly associated with activation of the pregnane X receptor (PXR), is also seen to increase upon exposure to PB. The results in this study favour CAR/PXR involvement with strong increases in the expression of *Cyp2b10* mRNA (particularly in males at lower doses than in females) along with mild positive responses in *Cyp3a11* expression. PROD and BROD liver enzyme activities, which give a measure of Cyp2b enzyme induction, concur with these results and were significantly elevated in both male and female mice at all doses and time-points analysed (range of 2.56 to 9.49-fold).

Involvement of the Aryl hydrocarbon receptor: The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor involved in the regulation of several genes, including those for xenobiotic-metabolizing enzymes such as cytochrome P450 *Cyp1a1* and *Cyp1b1*. Evaluation of the AhR-related EROD liver enzymatic activity showed a less than mediocre, but statistically significant increase (none greater than 2.3-fold; 33.30 pmol/min/mg protein), which may be associated with the large induction of Cyp2b enzyme (maximum increase of 9.49-fold). Investigations using β-napthoflavone, a prototypical AhR agonist, report far greater levels of elevated EROD activity (in excess of 1500pmol/min/mg protein). The results presented in the present report suggest that sulfoxaflor is not an agonist of AhR in the mouse.

Involvement of the Peroxisome proliferator-activated receptor (alpha): Liver *Cyp4a10* mRNA levels were examined to investigate any possible involvement of peroxisome proliferation activity by sulfoxaflor in mice. *Cyp4a10* levels were unchanged in males and reduced by up to 5.6 fold in female mice. These results also concur with measurements carried out on samples from the 28 day and 90 day mouse studies. PPARα activation is not thought to be an important mediator of liver effects with exposure to sulfoxaflor.

Hepatocellular proliferation: Liver proliferation following sulfoxaflor exposure was examined by Ki-67 immunohistochemical staining to identify proliferating hepatocytes in addition to BrdU incorporation. BrdU incorporation confirmed significant proliferation in both the centrilobular and midzonal regions of high dose males (750ppm), at the lower dose of 500ppm only the centrilobular region was significantly labelled. Females exposed to 1000 and 1500ppm showed increased proliferation in all three zones. To complement the BrdU analysis, the same samples were analysed by Ki-67 immunohistochemical staining. In males exposed to 500 and 750ppm, significant induction of centrilobular proliferation was observed, however no induction was noted in the midzonal region of 750ppm-treated males (positive when examined by BrdU). In females, there were no statistically identified increases in proliferation at any dose or zone, whereas BrdU showed increased proliferation in both doses and in all three liver regions. The differences in proliferation indices of Ki-67 and BrdU are a result of the analytical window that is measured by each method. BrdU is incorporated into the DNA of proliferating cells and is cumulative over time, while Ki-67 measures only the cells that have actively replicated at the time of animal sacrifice. It may be assumed that the majority of hepatic proliferation occurred prior to Day 7 of exposure. Studies examining liver proliferation in PB exposed rodents have shown significant increases by 7 days focused mainly in the centrilobular region. The observed proliferation in this study does not appear to be induced by cytotoxicity, but rather through a mitogenic mechanism resulting in increased cell number and organ size. These results suggest that the hepatocellular proliferation induced following sulfoxaflor exposure is similar to that observed following PB exposure.

The proliferation analysis (as measured by Ki-67) of the 28 and 90-day sulfoxaflor exposure did not identify any statistically significant increases in proliferation at the doses tested. In

males, after 28 days of exposure of 300ppm sulfoxaflor there were no differences when compared to control, however, in males exposed to 750ppm for 90 days there was a statistically identified decrease in proliferation in the centrilobular region. Similarly, females exposed to 1500ppm sulfoxaflor for 28 days had a decrease of Ki-67 positive cells in the periportal region and females treated with 1500ppm for 90 days showed decreased proliferation in the midzonal region. Together, these 28 and 90-day Ki-67 observations do not show a consistent lobular response and may not be treatment related, however they do show that sulfoxaflor is not inducing a prolonged hepatocellular proliferative response in contrast to the sustained induction of *Cyp2b10* gene expression and enzyme activity.

Based upon these results, increased liver weight in mice administered dietary sulfoxaflor appears to be similar to the action of phenobarbital, as evidenced by the CAR and PXR-related molecular, enzymatic, and proliferative responses.

Study 4: Mechanism of action Study: Mouse strain suitability. (DAR Section B.6.5.3.4.)

In previous studies, limited targeted gene expression data was generated in the Crl:CD1(ICR) mouse strain to support the hypothesis that sulfoxaflor acts through a phenobarbital (PB)-like mode of action (MoA) involving activation of the CAR receptor. The study by Elcomb, (section B.6.5.3.4; Elcomb, 2010) seeks to validate the suitability of an alternate mouse strain (C57BL/6J) with respect to liver enzyme induction, gene expression and proliferative responses to dietary sulfoxaflor. A more comprehensive investigation into the role of the CAR/PXR receptors can then be studied with the use of CAR/PXR knockout and humanised mice but these experimental models are only available in the C57BL/6J strain.

The administration of sulfoxaflor at dietary concentrations of 750ppm and 1500ppm to C57BL/6J male mice for 7 days increased absolute and relative liver weights by17% and 28% (absolute) and 17% and 40% (relative) respectively. This suggests that hepatocyte hypertrophy or hyperplasia had occurred. The absence of biologically significant changes in core hepatic parameters indicated necrosis was absent. Sulfoxaflor behaved as a phenobarbital-like inducer. This was demonstrated by the induction of total cytochrome P450, markedly increased PROD and BROD (Cyp2b selective substrates) activity along with a more modest increase in BQ activity (Cyp3a selective substrate). This phenobarbital-like induction was confirmed with increased expression of Cyp3a11 and Cyp2b10 mRNA demonstrated by RT-PCR, and by Western blotting data showing increases in Cyp3a11 and Cyp2b10 proteins. The results support the use of this alternate mouse strain (C57BL/6J) with respect to liver enzyme induction, gene expression and proliferative responses to dietary sulfoxaflor and allow the valid use of transgenic animals using double humanised PXR and CAR (hPXR/hCAR), and double knockout PXR and CAR (PXRKO/CARKO) genotypes based on the wild-type (WT) C57BL/6J mouse strain.

Report: B. M. Elcombe. (2010). XDE-208: A Study to Characterize the Induction Profile of XDE-208 in the Livers Of C57BL/6J Mice. CXR Biosciences Ltd., James Lindsay Place, Dundee Technopole, Dundee, DD1 5JJ and Medical School Resource Unit (MSRU), Dundee University, Dundee, DD1 9SY.

Report No.: Study ID: CXR0821. DECO HET DR-0404-3134-116

Dates: May 2009

Guidelines: Non-guideline.

GLP: No. However, all experiments were done according to GLP standards.

Deviations: None. This is acceptable as a basic though non-guideline short term MoA study, it is considered supplementary to the long-term chronic / carcinogenicity studies and critical to ensuring that data from an extensive study using C57BL/6J CAR/PXR knockout and humanised mice is comparable with the data generated in previous studies which utilised CD1 mice exposed to sulfoxaflor.

Deficiencies: Yes in that it would have been a more complete study to characterise the hepatomegaly with liver histopathology data because this effect is typically characterised by hepatocellular hypertrophy and hyperplasia in the short term.

Executive Summary: In previous studies, limited targeted gene expression data has been generated in the Crl:CD1(ICR) mouse strain to support the hypothesis that sulfoxaflor acts through a phenobarbital (PB)-like mode of action (MoA) involving activation of the CAR receptor. The present study seeks to validate the suitability of an alternate mouse strain (C57BL/6J) with respect to liver enzyme induction, gene expression and proliferative responses to dietary sulfoxaflor. A more comprehensive study of the role of the CAR/PXR receptors can be studied with the use of CAR/PXR knockout and humanised mice but these experimental models are only available in the C57BL/6J strain.

Sulfoxaflor was administered in the diet to 5 male C57BL/6J mice per dose group at dose levels of 0, 750, or 1500 ppm (equivalent to 0, 160, and 310 mg/kg/day respectively) for 7 days. The primary endpoints examined in this study included daily clinical observations, body weights, body weight gain, feed consumption, serum clinical chemistries, focused gene expression and protein quantification, liver cytochrome P450 enzyme activity, and hepatocellular proliferation. Males only were selected as they are more sensitive to the effects of sulfoxaflor. The dose levels selected were based upon previous studies in CD1 mice.

Sulfoxaflor administration resulted in hepatomegaly. There was a treatment-related increase in absolute and relative liver weights following seven days of exposure to sulfoxaflor. There was no evidence of hepatotoxicity at any dose level. Treated animals did have raised plasma ALT levels, there was a dose-dependent and statistically significant increase in ALT (< 2-fold in the high dose group relative to controls) but it is not considered toxicologically significant. There were also minor increases in both AST and triglycerides for the high dose group alone with little to no change in cholesterol levels amonst all dose groups. Cytochrome P450 activity increased with sulfoxaflor dose. Administration of sulfoxaflor at 750ppm and 1500ppm elicited a 3- to 5- fold increase in total hepatic P450, respectively, a 33-fold increase in PROD activity at both concentrations, a 47- and 82- folding increase in BROD activity, respectively, and a 4-and 7-fold increase in BQ activity, respectively

Elevations in *Cyp2b10* and *Cyp3a11* gene expression levels were observed. The strongest response was associated with the expression of *Cyp2b10* mRNA, going from indeterminant (i.e. very low) levels in controls to a positive dose response increment of 9-fold between the 2 doses tested. Unlike *Cyp2b10*, *Cyp3a11* is constitutively expressed in this strain of mouse (C57B1/6J), and results can be expressed as a relative fold change over control values. Administration of 750 and 1500 ppm XDE-208 resulted in a 2.4-and 5.6-fold increase in *Cyp3a11* relative to controls. Sulfoxaflor induced gene expression data was confirmed by investigation of the resultant gene products, i.e. proteins via sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting.

Overall, the data supports inter-strain comparisons to data previously obtained from studies in

CD1 mice and suggests an involvement of the CAR/PXR nuclear receptor system in the consequent liver effects seen with sulfoxaflor exposure.

Materials and Methods

Materials:

1	**Test Material:**	Sulfoxaflor
	Synonyms:	XDE-208; (N-(Methyloxido(1-(6-(trifluoromethyl)-3-pyridinyl)ethyl)-λ^4-sulfanylidene)-cyanamide); [1-(6-Trifluoromethylpyridin-3-yl)ethyl](methyl)-oxido-λ^4-sulfanylidenecyanamide; Sulfoximine; X11422208; XR-208.
	Description:	White Solid
	Lot/Batch #:	Lot # E2162-34, TSN003725-0001.
	Purity:	95.6% (w/w); as two diastereomers in 48.4 / 47.4% ratio
	Contaminants:	
	CAS #:	946578-00-3
2	**Vehicle:**	LabDiet Certified Rodent diet #5002 (PMI Nutrition International, St. Louis, Missouri, US)
	Dose	Ingested via the oral (dietary) route: Time-weighed average doses were: Males; day7: 0, 160, 310 mg/kg body weight/day.
3	**Test Animals:**	
	Species:	Mouse
	Strain:	C57BL/6J
	Age/weight at study initiation:	6-8 weeks / no data supplied
	Source:	Harlan UK Limited, Shaw's Farm, Blackthorn, Bicester, Oxon, England, OX25 1TP.
	Housing:	After assignment, mice were housed 5 per cage on sawdust in solid-bottom, individually ventilated polypropylene cages.
	Feed and Water:	RM1 powdered diet (Special Diet Services Ltd., Stepfield, Witham, Essex, UK) *ad libitum*. Municipal water *ad libitum*.
	Environmental conditions:	**Temperature:** 19 – 23°C **Humidity:** 40-70% **Air Changes:** 14-15 times/hour **Photoperiod:** 12-hour light/dark
	Acclimation period:	The mice were acclimatised for a period of 5 days before use. No animals were excluded from the study. Environment enhancing materials, tubes and paper bedding, were used during this study.

Study Design:

In life dates: Test material administration for all animals began on 13th May 2009. Mice were necropsied on 20th May 2009, (day 7 of treatment).

Animal assignment and treatment groups: Before administration of test material began, animals were stratified by body weight and then randomly assigned to treatment groups. Groups of five male C57BL/6J mice were fed diets supplying 0, 750, and 1500ppm sulfoxaflor for 7 days to obtain liver weight, clinical chemistry, targeted gene expression, and

enzyme activity information. An experimental card was placed on each cage and to show the project code, treatment group, study number, sex and individual numbers of the mice within. These cards were color coded to correlate with the treatment group.

Diet preparation and analysis: The sulfoxaflor powdered RM1 diet was prepared without purity correction. Diet was analyzed for achieved concentration and homogeneity by extraction and high performance liquid chromatography with negative ion electrospray ionization and mass spectrometry detection (HPLC/-ESI/MS). The mean concentration for each dose level ranged from 94% to 93% of targeted concentrations in the mid and high dose groups repectively, indicating acceptable concentrations of sulfoxaflor. The homogeneity of sulfoxaflor in the diets was described as "deemed homogenous", no other details available.

Statistics: Statistical comparisons between sulfoxaflor-treated and their respective control groups were undertaken for all numerical data sets using a 2-tailed Students' t-test.

Methods:

1. Observations: Prior to the start of the study, all mice were observed to ensure that they were physically and behaviorally normal. Each mouse was observed at least once daily during the study. Clinical abnormalities of individual animals were recorded in the Study Diary.

2. Body weight: The bodyweight of each mouse was recorded at the start of the study, each day of the study and on the day of termination.

3. Food consumption and compound intake: Feed consumption was determined for all animals by weighing feed containers at the start and end of a measurement cycle. The compound intake was calculated using test material concentrations in the feed, actual body weights (BW) and measured feed consumption data.

4. Clinical Chemistry: Markers of liver damage, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured in all available plasma samples. Liver-related plasma biomarkers (total cholesterol – CHOL and triglycerides – TG, were also investigated in all available plasma samples in order to characterise basal hepatic function. Liver was weighed and scissor-minced in ice-cold 1.15% (w/v) KCl prior to subcellular fractionation. Hepatic microsomes were isolated and stored at approximately -70°C until required for immunoblotting and cytochrome P450 activity assays. The protein content of the liver microsomes was determined in aqueous solutions using a modification of the method of Lowry et al., (1951) and bovine serum albumin as reference standard. Expression of *Cyp2b10* and *Cyp3a11* was carried out by SDS-PAGE and immunoblotting, using liver microsomes.

5. Targeted Gene Expression: Two pieces of liver, approximately $5mm^3$ were removed from the left lobe for TaqMan® analysis. These liver pieces were placed in the same cryovial, flash frozen in liquid nitrogen and then stored at approximately -70ºC until required. cDNA was synthesised from all available RNA samples and TaqMan analysis was performed using primers specific for *Cyp2b10* and *Cyp3a11* (Assay-on-demand kits, Cat # Mm00456591_ml and Mm00731567_m1, Applied Biosystems, respectively). Murine β-actin was used as the internal standard (Assay-on-demand kit, Cat # Mm00607939_sl, Applied Biosystems). Data was analysed by generation of CT and delta CT values for all genes.

The following genes were selected to address whether sulfoxaflor induces a phenobarbital-like gene expression response (both CAR and PXR mediated): *Cyp2b10, Cyp3a11*.

6. Liver Metabolic Enzyme Activities: The hepatic microsomal activity of *Cyp2b* (pentoxyresorufin-O-dealkylation, PROD; and benzyloxyresorufin-O-dealkylation, BROD) were measured. *Cyp3a11* activity was measured as the O-debenzylation of benzyloxyquinoline (BQ). In addition, the total cytochrome P450 content of the liver microsomal fraction was also determined.

7. Sacrifice and pathology: On the day of termination the mice were weighed and transferred to the post mortem room. The mice were killed by exposure to a rising concentration of CO_2. Venous blood was taken by cardiac puncture and dispensed into lithium/heparin coated tubes. The tubes were mixed on a roller for 10 min then cooled on ice. Red blood cells were removed by centrifugation (2,000 rpm for 10 min at 8 – 10 °C) and the supernatant (plasma) was transferred to a second tube and stored at approximately -70°C until required for clinical chemistry analysis.

Results and Discussion

Observations

Clinical signs of toxicity:

There were no clinical findings due to active substance exposure during this study. All mice survived until scheduled necropsy.

Mortality:

None.

Body weight and body weight gain

Administration of dietary sulfoxaflor to mice for 7 days had an impact on body weight. Figure 6.5.3.4-1 notes the similar body weights throughout the experiment for the control and lower dietary groups but shows an approximately 10% difference in body weight for the high dose animals relative to controls. Terminal body weights were depressed nearly 10% in the high dose group (21.2g *vs* 21.1g *vs* 19.2g for the control, 750ppm and 1500ppm groups respectively) and this is corroborated by reductions in feed consumption. This effect, while treatment related, is thought to be a consequence of palatability issues with the feed rather than a toxicological one. In several previous studies (B.6.3.1/3a study 060523; B.6.3.1/2, study 061170; B6.3, study 060488; B6.5.3.2, study 070339) using large dietary concentrations of sulfoxaflor, lower body weights and decreased body weight gain were associated with treatment-related differences in feed consumption and were attributed to a decreased palatability of the rodent feed due to its content of sulfoxaflor.

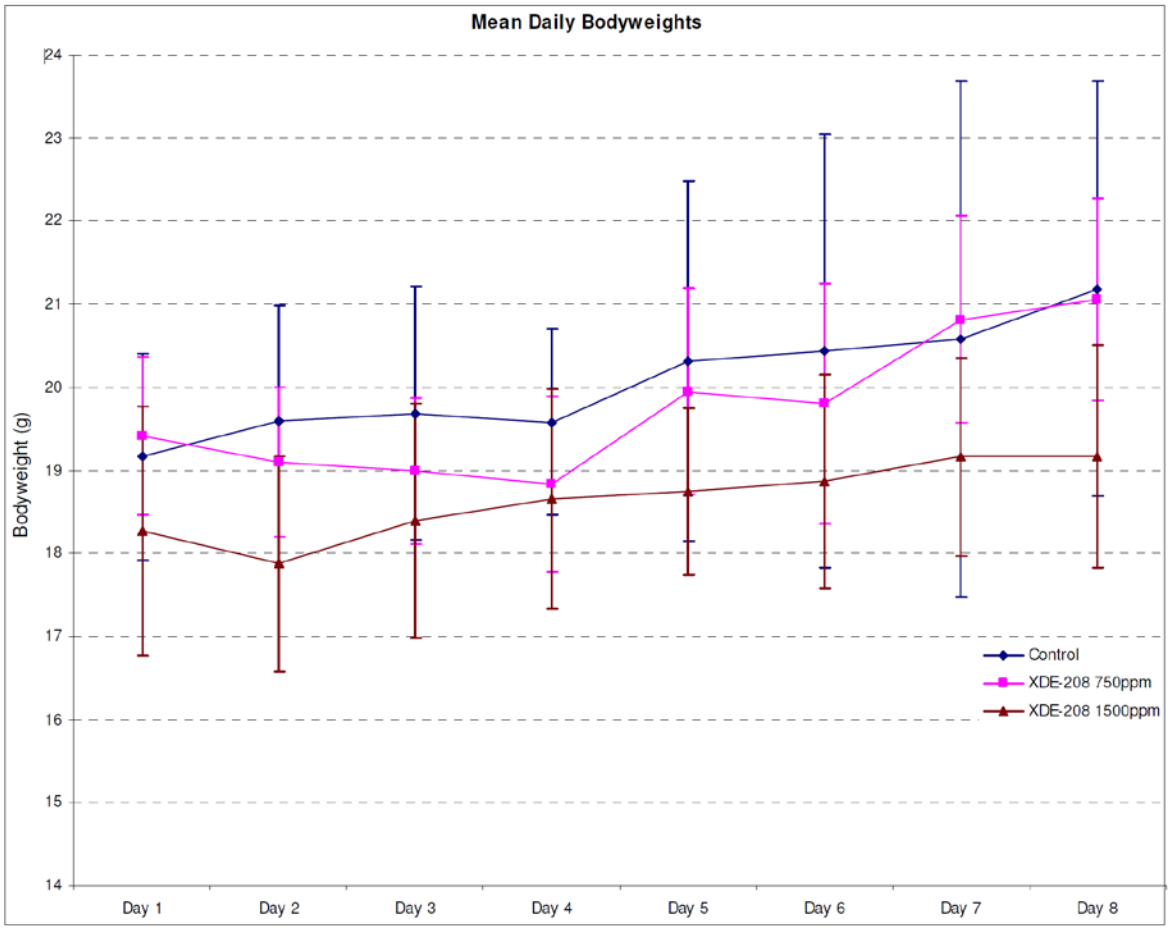


Figure 4.10.3.1.Study 4.1 (DAR Figure 6.5.3.4-1): mean daily bodyweights for the duration of the study. Controls and animals in the 750ppm diet group exhibit similar body weight gain after an initial lag up to day 4. The high dose animals show a distict trend for lower body weight gain relative to the other groups. Visual inspection suggests high dose animals have bodyweights of between 5% and 10% less than controls. Previous studies in rodents have observed palatability issues with diets containing high concentrations of sulfoxaflor.

Food consumption and compound intake

Food consumption was decreased in both treatment groups. In table 6.5.3.4-1, the mean daily diet consumed (g/mouse/day) was decreased 27% and 34% in the 750ppm and 1500ppm dose groups, respectively, relative to control. The mean ingested doses from the 2 treatment groups of 750ppm and 1500ppm sulfoxaflor, calculated for the duration of the study, were 160.66 ± 17.68 and 310.70 ± 33.13 mg sulfoxaflor/kg body weight/day, respectively.

Table 4.10.3.1.Study 4.1 (DAR Table 6.5.3.4-1): Summary of the daily Mean Diet Consumed

Parameter	Control	750ppm	1500ppm
Diet (g/mouse/day)	6.33 ± 3.04[a]	4.59 ± 2.42	4.17 ± 2.00
Diet (g/kg bwt/day)	310.01 ± 140.87	227.88 ± 110.02	223.36 ± 106.64

Clinical pathology

Clinical Chemistry:

The clinical chemistry parameters measured were triglycerides (TG), cholesterol (CHOL), alanine aminotransferase (ALT), and aspartate aminotransferase (AST). Triglycerides were slightly elevated in high dose males (36%) although the difference from control was not statistically significant. (See table 6.5.3.3-2) This was considered treatment-related because increases in triglycerides have been noted in female rats given 3500ppm for 28 days or 1500ppm or greater for 90 days. There is a dose-dependent and statistically significant increase in ALT but it is not considered of toxicological significance. All individual mouse ALT values were within the performing lab's historical range for this strain of mouse (mean ± sd, 40 ± 14; range, 11 – 86; n = 128). There were little to no changes of any significance in AST and cholesterol blood levels.

Table 4.10.3.1.Study 4.2 (DAR Table 6.5.3.4-2): Summary of clinical chemistry data (n = 5, all doses).

Parameter	0	750	1500
TG mmol/L	1.07 ± 0.28	1.08 ± 0.10	1.46 ± 0.29
CHOL mmol/L	2.71 ± 0.18	2.49 ± 0.08*	2.48 ± 0.26
ALT U/L	20.02 ± 1.79	28.30 ± 3.60**	38.60 ± 4.45***
AST U/L	71.80 ± 40.71	64.90 ± 26.95	82.84 ± 26.07

Values are Mean ± SD. A Student's t-test (2-sided) was performed on the results; *statistically different from control $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Sacrifice and Pathology

Organ weights:

Final body weights of high dose males were nearly 10% less when compared to their respective controls. Hepatomegaly was evident. Liver weights were increased in both the mid and high dose animals by 17% and 28% relative to controls respectively (table 6.5.3.4-3). Similarly, relative liver weights were also increased; from 17% in the mid dose group to 40% in the high dose group, while females from the 1000 or 1500 ppm had increases of 39% and 43% respectively.

Table 4.10.3.1.Study 4.3 (DAR Table 6.5.3.4-3): Mean terminal body and liver absolute and relative weights (n = 5).

Parameter	0	750	1500
Body weight (g)	21.19 ± 2.49	21.05 ± 1.22	19.16 ± 1.34
Liver wt. (g)	1.11 ± 0.09	1.29 ± 0.09*	1.42 ± 0.14**
Rel. liver wt. (g/100g)	5.27 ± 0.56	6.14 ± 0.32*	7.38 ± 0.27***

Values are Mean ± SD. A Student's t-test (2-sided) was performed on the results; *statistically different from control $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Targeted gene expression, enzyme activity and protein content

1. CAR and PXR associated events:

To investigate if sulfoxaflor exposure resulted in a gene expression pattern similar to that observed with phenobarbital exposure, *Cyp2b10* and *Cyp3a11* were measured by quantitative RT-PCR using specific primers and probes, with murine β-actin as the reference gene. Additionally, Cyp2b enzyme activity was evaluated by benzyloxyresorufin (BROD) and pentoxyresorufin (PROD) O-dealkylase activities while benzylquinoline debenzylation activity (BQ) is used for the quantification of mouse *Cyp3a11* activity. *Cyp2b10* gene induction, considered to be the prototypical gene response following PB exposure through activation of the constitutive active/androstane nuclear receptor (CAR), was induced but by how much is unclear. It is transcribed at a very low level in the controls and a direct comparison of the relative increase in gene induction was not possible from the data. However, sulfoxaflor treatment is clearly seen to induce *Cyp2b10* in both treatment groups with a 9-fold difference in expression levels between the 750ppm and 1500ppm groups (table 6.5.3.4-4). This induction is confirmed by Western blot analysis showing increased levels of protein in both treatment groups. *Cyp3a11*, which is also associated with PB-like gene expression through the pregnane X nuclear receptor (PXR), and is also expressed in a basal constituitive manner, was also elevated in both treatment groups. Mean cycle times for *Cyp3a11* are: 25.8 for controls, 24.6 for the 750ppm group and 23.0 for the high dose group. These represent a 2.4-fold change for the 750 ppm treatment over control and a 5.6-fold change for the high dose group over controls (table 6.5.3.4-4).

Table 4.10.3.1.Study 4.4 (DAR Table 6.5.3.4-4): Targeted gene expression expressed as change in mean Ct value compared to control.

		Male			
study	**dose (ppm)**	***Cyp2b10***	***rel. fold change***	***Cyp3a11***	***rel. fold change***
7 day mouse	control (0)	> **35**	--	25.8	--
	750ppm	31.4	--	24.6	2.4
	1500ppm	26.8	9.2	23.0	5.6

Summary of TaqMan® analysis of murine *Cyp2b10* and *Cyp3A11* mRNA. The greater the Ct value, the lower the mRNA expression. Ct values > 35 indicates mRNA levels below the limits of detection for the assay, ie little expression taking place.

2. Cytochrome P450 enzyme analysis:

Both PROD and BROD liver enzyme activities, which are associated with CAR activation and give a measure of Cyp2b enzyme induction, were likewise significantly elevated in this study. Administration of sulfoxaflor at both dietary concentrations elicited similar but marked increases in PROD activity of approximately 33-fold after 7 days on the 2 respective diets (table 6.5.3.4-5). Sulfoxaflor elicited marked dose-dependent increases in BROD activity of approximately 47- and 82-fold for the lower and higher dose treatments respectively, after a short period of just 7 days. Benzylquinoline debenzylation (BQ) is used for the quantification of mouse Cyp3a11 activity. Administration of sulfoxaflor at 750ppm or 1500ppm elicited a small but significant dose-dependent increase in BQ activity of approximately 4- and 7-fold respectively following 7 days of treatment. Significant increases in hepatic total cytochromes P450 of approximately 3- and 5-fold were also observed.

Table 4.10.3.1.Study 4.5 (DAR Table 6.5.3.4-5): Cytochrome P450 enzyme activity and content with respect to treatment.			
Parameter	**Control**	**750ppm**	**1500ppm**
[1]PROD	5.75 ± 3.42	189.68 ± 66.14*** (↑) 33-fold	192.79 ± 31.81*** (↑) 33-fold
[1]BROD	36.4 ± 32.8	1713.8 ± 447.4*** (↑) 47-fold	2987.9 ± 245.5*** (↑) 82-fold
[2]BQ	0.96 ± 0.13	3.53 ± 0.71*** (↑) 4-fold	6.70 ± 0.59*** (↑) 7-fold
[3]Total P450	0.268 ± 0.136	0.775 ± 0.184** (↑) 3-fold	1.298 ± 0.126*** (↑) 5-fold

Values are mean ± sd. [1] pmols product/min/mg protein; [2]nmols product/min/mg protein; [3]nmols/mg protein. A Student's t-test (2-sided) was performed on the results; *statistically different from control with $p<0.05$; **$p<0.01$; *** $p<0.001$

3. Qualitative protein analysis – Western blotting of hepatice microsomes:

SDS-PAGE was performed on samples from all animals to separate the microsomal proteins, including the cytochrome P450 enzymes and Western blotting was performed to identify specific changes in the level of *Cyp2b10* and *Cyp3a11* translated protein. Figure 6.5.3.4-2 clearly shows increased levels of Cyp2b10 and Cyp3a11 protein in a dose dependent manner, in response to sulfoxaflor treatment.

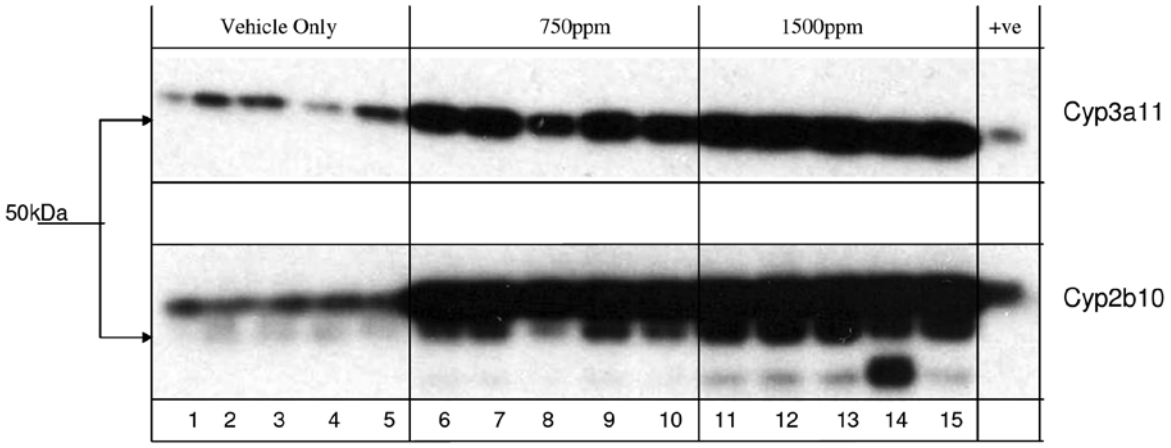


Figure 4.10.3.1.Study 4.2 (DAR Figure 6.5.3.4-2): Western blot of both Cyp2b10 and Cyp3a11 from microsomes isolated from each animal on the study. Lane number corresponds to each individual animal. Protein of 1µg was loaded for each sample onto a 7.5% SDS page gel. Recombinant proteins were added as positive controls (Cyp3a11 was loaded at 0.025pmol. Cyp2b10 was loaded at 0.01pmol).

Conclusions

Due to similarities in PB-like gene expression profiles and related endpoints (such as increased liver weight), it is thought that the hepatocyte and liver effects seen with exposure

to sulfoxaflor are mediated through CAR/PXR nuclear receptor activation. Previous studies have investigated these effects using both the Fisher F344 rat and the Crl:CD1(ICR) mouse strains. The present study supports the use of an alternate mouse strain (C57BL/6J) with respect to liver enzyme induction, gene expression and proliferative responses to dietary sulfoxaflor. The administration of sulfoxaflor at dietary concentrations of 750ppm and 1500ppm to C57BL/6J male mice for 7 days increased absolute and relative liver weights by17% and 28% (absolute) and 17% and 40% (relative) respectively. This suggests that hepatocyte hypertrophy or hyperplasia had occurred. The absence of biologically significant changes in core hepatic parameters indicated necrosis was absent. Sulfoxaflor behaved as a phenobarbital-like inducer. This was demonstrated by the induction of total cytochrome P450, markedly increased PROD and BROD (Cyp2b selective substrates) activity along with a more modest increase in BQ activity (Cyp3a selective substrate). This phenobarbital-like induction was confirmed with increased expression of *Cyp3a11* and *Cyp2b10* mRNA demonstrated by RT-PCR, and by Western blotting data showing increases in Cyp3a11 and Cyp2b10 proteins.

Induction of *Cyp2b* typically would suggest activation of the constitutive androstane receptor (CAR) while induction of *Cyp3a* may also suggest at least some involvment of the pregnane-X-receptor (PXR). However, there is often cross reactivity or overlapping activity between the 2 transcription activators and low levels of Cyp3a induction are often mediated by activation of CAR alone (or in concert with the retinoid X receptor – RXR). Recent studies where phenobarbital was administered to knockout CAR and knockout PXR mice have suggested that, at least in mice, PXR activation is not always required for the induction of *Cyp3a* and that such induction may be mediated solely by CAR (albeit at low levels relative to *Cyp2b* induction) and not PXR (*Scheer et al., 2008*).

Based upon these results, increased liver weight in C57BL/6J mice administered dietary sulfoxaflor appears to be similar to the action of phenobarbital, as evidenced by the CAR and PXR-related molecular and enzymatic responses and is comparable to those seen in other rodent species and genetic strains.

IV. References

Scheer, N., Ross, J., Rode, A., Zevnik, B., Niehaves, S., Faust, N., and Wolf, C.R. (2008). A novel panel of mouse models to evaluate the role of human pregnane X receptor and constitutive androstane receptor in drug response. J Clin Invest 118:322 3239.

Study 5: MoA Study: Mouse/C57Bl/6J WT, Humanised and KO PXR/CAR transgenic models. (DAR Section B.6.5.3.5.)

To further investigate the role of the nuclear receptors CAR/PXR, additional experiments were conducted at CXR Biosciences in Dundee, Scotland using two genetically modified mouse models (section B.6.5.3.5; Ross, 2010). The first was a 'knock-in' mouse that contained human receptors for PXR and CAR (hereafter referred to as hPXR/hCAR) while the second mouse model was null, or knocked-out for both receptors (hereafter referred to as PXRKO/CARKO). Both nuclear receptors were investigated because sulfoxaflor and PB have been shown to interact with CAR and PXR in vitro (Geter et al., 2010; Lehmann et al. 1998).

Creation of the hPXR/hCAR mouse model required insertion of the human PXR and CAR genes into the exact location that these genes were found in the mouse genome. This ensures that the mouse gene is no longer present and the human DNA will be read when the cell

produces the PXR or CAR proteins (and all potential splice variants). In creating the PXRKO/CARKO, scientists started with the hPXR/hCAR mouse model and effectively removed the PXR and CAR genes from the mouse genome. This made a mouse that could not make either the PXR or CAR proteins, even if the cell needed them. Specifically, the hPXR/hCAR mice were generated by a 'knock-in' process that ensured expression of the human receptors controlled by the corresponding mouse promoters, thus deleting any endogenous gene function (Sheer et al., 2008). Both the humanised and KO mice appeared normal and could not be distinguished from wild-type (WT) C57BL/6J mice. They had normal survival rates and fertility, and pathological analysis on the livers were all normal (Ross, 2010).

The goal of the humanised and knock-out PXR/CAR mouse study was to determine: (1) if CAR/PXR mediate sulfoxaflor-induced hypertrophy and hyperplasia in mice; and (2) if the hPXR/hCAR mice shared a similar response as seen in the WT (C57BL/6J) mice (and hence the CD1 mouse) following sulfoxaflor exposure. In this study, groups of ten male mice of each strain were offered diets containing 0 or 750ppm sulfoxaflor for seven days. The results of this study showed that sulfoxaflor demonstrated greater activity towards mouse than human CAR and relatively weak activity towards the mouse and human PXR. WT C57BL/6J mice demonstrated all of the adverse liver effects as seen in the previous studies using CD-1 mice. In the PXRKO/CARKO mice, sulfoxaflor failed to induce any liver changes demonstrating that activation of these receptors are required to elicit the adverse liver effects seen following exposure in WT mice. Sulfoxaflor exposed hPXR/hCAR mice did develop slight liver hypertrophy and other changes, however, as for PB in humans, hepatocellular proliferation was not observed. This study demonstrated that sulfoxaflor, like PB, acts via a CAR-mediated MoA and that mice carrying the human PXR and CAR receptors did not exhibit hepatocellular proliferation.

With a PB-like MoA, hepatocellular proliferation is the key event separating a human non-cancer response from the rodent cancer response. When mice were examined in an 18-month cancer bioassay, males exposed to 750ppm and females to 1250ppm sulfoxaflor showed an increase in hepatocellular adenomas and/or carcinomas of 60 and 10% respectively. The higher incidence of tumours in male mice was expected as this was also seen in chronic rodent PB studies (Peraino et al., 1973, Rossi et al., 1977, Thorpe and Walker, 1973) and within the previous sulfoxaflor MoA studies, males were affected to a greater extent than females. Furthermore, since all of the key events required for CAR-associated rodent liver tumours were seen following exposure, it was clear that a liver tumourgenic response would be observed. However, there is no evidence that PB increases hepatocellular proliferation in humans and in the humanised PXR/CAR mouse, sulfoxaflor did not cause any increase in proliferation.

When rats were treated with sulfoxaflor for 24 months in the long-term carcinogenicity study, males exposed to 500ppm showed an increase in hepatocellular adenomas whereas females did not (750ppm). There were no treatment-related liver effects in males or females given 25 or 100ppm sulfoxaflor. The occurrence of tumours in male but not female rats again follows that seen in PB rodent cancer studies and in the rat MoA studies where males were affected to a greater extent than females. All of the key events required for CAR-associated rodent liver tumours have been seen following sulfoxaflor exposure, thus it was expected that a liver tumourgenic response would be observed. From these data, it is concluded that sulfoxaflor induces hepatocellular tumours in both the mouse and rat, but like phenobarbital, it acts via a CAR-mediated mode of action and would not be expected to be a human liver carcinogen.

Report: Ross, J. XDE-208 (2010): A Study To Investigate The Mode of Action For

Liver Effects Observed In Regulatory Toxicology Studies By Use of Dual Car-PXR Knockout And Humanised Mice.CXR Biosciences Ltd., James Lindsay Place, Dundee Technopole, Dundee, DD1 5JJ and Medical School Resource Unit (MSRU), Dundee University, Dundee, DD1 9SY.

Report No.: Study ID: CXR0867. DECO HET DR-0404-3134-112.

Dates: 2009

Guidelines: Non-guideline.

GLP: No. However, all experiments were done according to GLP standards.

Deviations: None. This is an acceptable though non-guideline short term MoA study, it is considered supplementary to the long-term chronic / carcinogenicity studies and critical to illustrating the roles of the CAR/PXR nuclear receptors in mediating the effects of sulfoxaflor on the rodent liver as well as accounting for species differences in liver response.

Deficiencies: None. General comments: no definitive distinction between CAR and PXR activities because double knockout mice and double humanised PXR-CAR mice were used in this study.

Executive Summary: In previous rodent studies hepatomegaly characterised by hepatocellular hypertrophy and hyperplasia in the short term, and, at high doses, hepatocellular tumours in the long term, is a feature of sulfoxaflor exposure. Limited targeted gene expression data indicates similarities to gene expression events promoted by phenobarbital which is known to activate the CAR receptor. So called “humanised” and knockout PXR and CAR mouse models have been utilised to investigate the effects of xenobiotics on the liver as it is wellknown that CAR/PXR are involved in the apparent species differences in the stimulation of the hyperplastic response. The CAR/PXR knockout models can be used to identify whether the mechanism of action is CAR/PXR-dependent and therefore potentially similar to the effects caused by phenobarbital. Phenobarbital is an example of a mouse non-genotoxic carcinogen that according to all reports so far, does not cause cancer in humans. The use of “humanised” CAR/PXR animals obliterates the proliferative or hyperplastic response normally seen in wild type animals exposed to phenobarbital and raises questions whether xenobiotics such as phenobarbital pose a hepatocarcinogenic hazard to humans.

The purpose of the study was to investigate: (1) if the constitutive androstane receptor (CAR) and pregnane X receptor (PXR) mediate sulfoxaflor-induced hypertrophy and hyperplasia in mice; and (2) if the human orthologs support these processes to a similar extent as the murine receptors. The mouse models used were wild type C57BL/6J (WT) mice, C57BL/6J mice null for PXR and CAR (PXRKO/CARKO) and C57BL/6J mice “humanised” for PXR and CAR (hPXR/hCAR). Sulfoxaflor was administered in the diet to 10 male rats of each strain at a dose level of 750ppm (equivalent to 115.6, 120.4 and 99.3mg sulfoxaflor/kg body weight/day, for WT, PXRKO/CARKO and hPXR/hCAR mice respectively) and 0 dose controls for 7 days. Parameters examined included: daily clinical observations, body weights, body weight gain, feed consumption, plasma clinical chemistries, focused gene expression, protein quantification, liver cytochrome P450 enzyme activity, hepatocellular proliferation using nuclear incorporation of BrdU, and liver histopathology.

There were no treatment-related clinical observations or effects on body weight or body weight gain in any strain of mouse. There were treatment-related increases in absolute (24%

and 9% respectively) and relative (25% and 12% respectively) liver weights in WT and hPXR/hCAR mice but not in the PXRKO/CARKO animals. In WT mice, sulfoxaflor treatment increased hepatocellular proliferation (approximately 4-fold) but no such changes in proliferation were seen in either the hPXR/hCAR or PXRKO/CARKO mice. Treatment-related hepatocyte hypertrophy was observed in WT and hPXR/hCAR mice while increased mitotic figures were observed only in WT mice (the knockouts failed to show either response).

Sulfoxaflor behaved as a phenobarbital-like inducer in WT mice (marked induction of total cytochrome P450, increased PROD and BROD, increased expression of *Cyp2b10* mRNA, and increases in Cyp2b10 protein. However, in the hPXR/hCAR under the same conditions, induction of Cyp2b10 activity, protein and mRNA was markedly less than observed in the WT animals following treatment with sulfoxaflor. Sulfoxaflor had no significant effect on *Cyp2b10* expression or catalytic activity in the genetic knock outs.

Similar sulfoxaflor-mediated *Cyp3a11* induction, as determined by BQ activity, RT-PCR and immunoblotting was observed in the "humanised" and WT mice, but was not seen in the PXRKO/CARKO mice.

The results suggest sulfoxaflor exhibits more activity towards the mouse CAR/PXR than the human CAR/PXR and that the CAR/PXR receptors are intimately tied into the liver response resulting from sulfoxaflor exposure. Additionally, the data show that the human CAR/PXR support sulfoxaflor-induced hypertrophy but not hyperplasia thus indicating species susceptibility differences due to the CAR/PXR genotype present.

Materials and Methods

Materials:

1	**Test Material:**	Sulfoxaflor
	Synonyms:	XDE-208; (N-(Methyloxido(1-(6-(trifluoromethyl)-3-pyridinyl)ethyl)-λ^4-sulfanylidene)-cyanamide); [1-(6-Trifluoromethylpyridin-3-yl)ethyl](methyl)-oxido-λ^4-sulfanylidenecyanamide; Sulfoximine; X11422208; XR-208.
	Description:	White Solid
	Lot/Batch #:	Lot # E2162-34, TSN003725-0001.
	Purity:	95.6% (w/w); as two diastereomers in 48.4 / 47.4% ratio
	Contaminants:	
	CAS #:	946578-00-3
2	**Vehicle:**	LabDiet Certified Rodent diet #5002 (PMI Nutrition International, St. Louis, Missouri, US)
	Dose	Ingested via the oral (dietary) route: Time-weighed average doses were: Males; day7: 0, and 99 – 120mg/kg body weight/day.

3	**Test Animals:**	
	Species:	Mouse
	Strain:	C57BL/6J: WT, hPXR/hCAR, PXRKO/CARKO
	Age/weight at study initiation:	8-10 weeks / no data supplied
	Source:	C57BL/6J (WT): Harlan UK Limited, Shaw's Farm, Blackthorn, Bicester, Oxon, England, OX25 1TP. hPXR/hCAR mice and PXRKO/CARKO mice: Taconic Farms, 273 Hover Avenue, Germantown, NY 12526, USA
	Housing:	After assignment, mice were housed 5 per cage on sawdust in solid-bottom, individually ventilated polypropylene cages.
	Feed and Water:	RM1 powdered diet (Special Diet Services Ltd., Stepfield, Witham, Essex, UK) *ad libitum*. Municipal water *ad libitum*.
	Environmental conditions:	**Temperature:** 19 – 23°C **Humidity:** 40-70% **Air Changes:** 14-15 times/hour **Photoperiod:** 12-hour light/dark
	Acclimation period:	The mice were acclimatised for a period of 5 days before use. No animals were excluded from the study. Environment enhancing materials, tubes and paper bedding, were used during this study.

Study Design:

1. In life dates: Test material administration for all animals began on 29th Sept 2009 for a duration of 7 days. Mice were necropsied on 7th Oct 2009.

2. Animal assignment and treatment groups: Before administration of test material began, animals were stratified by body weight and then randomly assigned to treatment groups. Groups of 10 male mice were fed diets supplying either 0 or 750ppm sulfoxaflor for 7 days with the intention to obtain liver weight, clinical chemistry, targeted gene expression, and enzyme activity information. An experimental card was placed on each cage and to show the project code, treatment group, study number, sex and individual numbers of the mice within. These cards were color coded to correlate with the treatment/genetic group.

3. Diet preparation and analysis: The sulfoxaflor powdered RM1 diet was prepared without purity correction. Diet was analysed for achieved concentration and homogeneity by extraction and high performance liquid chromatography with negative ion electrospray ionization and mass spectrometry detection (HPLC/-ESI/MS). The mean concentration for the 750ppm dose level was 98.6% of the targeted concentration indicating an acceptable concentration of sulfoxaflor was achieved. The homogeneity of sulfoxaflor in the diets was described as "deemed homogenous", no other details available.

4. Statistics: Statistical comparisons between sulfoxaflor-treated and their respective control groups were undertaken for all numerical data sets using a 2-tailed Students' t-test.

Methods:

1. Observations: Prior to the start of the study, all mice were observed to ensure that they were physically and behaviorally normal. Each mouse was observed at least once daily during the study. Clinical abnormalities of individual animals were recorded in the Study Diary.

2. Body weight: The bodyweight of each mouse was recorded at the start of the study, each day of the study and on the day of termination.

3. Food consumption and compound intake: Feed consumption was determined for all animals by weighing feed containers at the start and end of a measurement cycle. The compound intake was calculated using test material concentrations in the feed, actual body weights (BW) and measured feed consumption data.

4. Clinical Chemistry: Markers of liver damage, alanine aminotransferase (ALT), alkaline phosphatase (ALP) and aspartate aminotransferase (AST) levels were measured in all available plasma samples. Liver-related plasma biomarkers (total cholesterol – CHOL and triglycerides – TG, were also investigated in all available plasma samples in order to characterise basal hepatic function. Liver was weighed and scissor-minced in ice-cold 1.15% (w/v) KCl prior to subcellular fractionation. Hepatic microsomes were isolated and stored at approximately -70°C until required for immunoblotting and cytochrome P450 activity assays. The protein content of the liver microsomes was determined in aqueous solutions using a modification of the method of Lowry et al., (1951) and bovine serum albumin as reference standard. Expression of *Cyp2b10* and *Cyp3a11* was carried out by SDS-PAGE and immunoblotting, using liver microsomes.

5. Targeted Gene Expression: Two pieces of liver, approximately 5mm^3 were removed from the left lobe for TaqMan® analysis. These liver pieces were placed in the same cryovial, flash frozen in liquid nitrogen and then stored at approximately -70ºC until required. cDNA was synthesised from all available RNA samples and TaqMan analysis was performed using primers specific for *Cyp2b10* and *Cyp3a11* (Assay-on-demand kits, Cat # Mm00456591_ml and Mm00731567_m1, Applied Biosystems, respectively). Murine β-actin was used as the internal standard (Assay-on-demand kit, Cat # Mm00607939_sl, Applied Biosystems). Data was analysed by generation of CT and delta CT values for all genes. The following genes were selected to address whether sulfoxaflor induces a phenobarbital-like gene expression response (both CAR and PXR mediated): *Cyp2b10*, *Cyp3a11*.

6. Measurement of liver cell proliferation: Two samples of each liver, were taken – one from the left lobe and one from the median lobe. These were placed in a 25ml plastic sample pot containing approximately 20ml of 10% neutral buffered formalin (NBF) for histological analysis. The remaining liver was weighed and scissor-minced in ice-cold 1.15% (w/v) KCl prior to homogenisation and subcellular fractionation. A 0.5 cm section of the proximal small intestine (duodenum) was removed and processed in a similar manner to the liver using 10% NBF. Duodenum acts as a positive immunohistochemical control since its epithelial lining is continuously proliferating to replace lost enterocytes. Incorporation of 5-bromo-2'-deoxyuridine (BrdU; a structural analog of thymidine), into nuclear DNA was used as a surrogate marker of cell proliferation. One day prior to exposure to the sulfoxaflor treated diets, all study animals were implanted with mini-osmotic pumps (Model 2001; Alzet Corporation, Palo Alto, California). Mice were continuously infused with BrdU via the implanted osmotic pumps filled with a 15mg/ml solution of BrdU in phosphate buffered

saline (pH 7.4). Levels of hepatocyte S-phase DNA synthesis were determined using BrdU immunohistochemistry.

7. Liver Metabolic Enzyme Activities: The hepatic microsomal activity of *Cyp2b* (pentoxyresorufin-O-dealkylation, PROD; and benzyloxyresorufin-O-dealkylation, BROD) were measured. *Cyp3a11* activity was measured as the O-debenzylation of benzyloxyquinoline (BQ). In addition, the total cytochrome P450 content of the liver microsomal fraction was also determined.

8. Sacrifice and pathology: On the day of termination the mice were weighed and transferred to the post mortem room. The mice were killed by exposure to a rising concentration of CO_2. Venous blood was taken by cardiac puncture and dispensed into lithium/heparin coated tubes. The tubes were mixed on a roller for 10 min then cooled on ice. Red blood cells were removed by centrifugation (2,000 rpm for 10 min at 8 – 10 °C) and the supernatant (plasma) was transferred to a second tube and stored at approximately -70°C until required for clinical chemistry analysis.

Results and Discussion

Observations

Clinical signs of toxicity:

There were no clinical findings due to active substance exposure during this study. All mice survived until scheduled necropsy.

Mortality:

None.

Body weight and body weight gain

Administration of sulfoxaflor had little effect on the bodyweight (table 6.5.3.5-1) over the 7 day period. Similarly body weight gains were similar in all groups with no change of biological significance.

Table 4.10.3.1.Study 5.1 (DAR Table 6.5.3.5-1): Body weights and body weight gains for all groups (n = 10, per group).

Dose (ppm)	WT		PXRKO/CARKO		hPXR/hCAR	
	0	750	0	750	0	750
Initial wt (g)	21.2±0.9	22.2±1.7	23.9±1.3	24.6±1.5	25.3±1.2	24.3±1.4
day 8 (g)	22.4±1.0	22.2±1.8	24.1±1.3	24.7±1.4	25.5±1.4	24.8±1.5
*% bw gain**	*6%*	*< 1%*	*< 1%*	*< 1%*	*< 1%*	*2%*

*body weight (gain) % difference from 0 controls. Values are means ± sd.

Food consumption and compound intake

Food consumption was not significantly different amongst the different treatment groups (table 6.5.3.5-2). The mean ingested doses from the 750ppm treatment groups of WT,

PXRKO/CARKO and hPXR/hCAR mice, calculated for the duration of the study, were 115.58, 120.38 and 99.29 mg sulfoxaflor/kg body weight/day, respectively.

Table 4.10.3.1.Study 5.2 (DAR Table 6.5.3.5-2): Summary of the daily Mean Diet Consumed (n = 10, per group).

Dose (ppm)	WT		PXRKO/CARKO		hPXR/hCAR	
	0	750	0	750	0	750
Diet (g/mouse/day)	3.50	3.37	3.30	4.15	3.32	3.39
Diet (g/kg bw/day)	156.85	156.19	139.03	162.67	131.58	134.17

Clinical pathology

Clinical Chemistry:

The clinical chemistry parameters measured were triglycerides (TG), cholesterol (CHOL), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and aspartate aminotransferase (AST). Triglycerides were only elevated in WT animals (43%) although the difference from control was not statistically significant (See table 6.5.3.5-3). There is a slight increase (26%) in WT ALT enzyme activity (not biologically significant) with little to no change in the PXRKO/CARKO or hPXR/hCAR groups. ALP is raised slightly (24%) in the hPXR/hCAR group (not biologically significant), and decreased in the other groups. There were little to no changes of any significance in AST and cholesterol blood levels.

Table 4.10.3.1.Study 5.3 (DAR Table 6.5.3.5-3): Summary of clinical chemistry data (n = 10 per group).

parameter	WT		PXRKO/CARKO		hPXR/hCAR	
	0	750ppm	0	750ppm	0	750ppm
TG mmol/L	1.03 ± 0.28	1.47 ± 0.45	1.10± 0.28	1.11± 0.18	1.54 ± 0.33	1.60 ± 0.32
CHOL mmol/L	2.95 ± 0.74	2.84 ± 0.48	2.35 ± 0.22	2.26 ± 0.21	2.85 ± 0.24	2.62 ± 0.19*
ALT U/L	29.0 ± 5.0	35.0 ± 8.1	32.4 ± 7.8	31.6 ± 6.0	32.2 ± 3.8	33.3 ± 4.4
ALP (U/L)	89.3 ± 12.5	73.0 ± 20.4	66.8 ± 10.6	56.0 ± 13.1	62.6 ± 7.5	77.9 ± 13.1**
AST (U/L)	85.8 ± 29.1	108.5 ± 39.3	130.9 ± 34.4	152.5 ± 114.1	105.7 ± 52.9	94.2 ± 26.1

Values represent mean ± sd. A Student's t-test (2-sided) was performed on the results; *statistically different from control $p<0.05$; **$p<0.01$. CHOL = cholesterol, TG = triglycerides.

Sacrifice and Pathology

Organ weights:

Absolute liver weights were increased in WT mice (19%) but not in the transgenic animals following exposure to sulfoxaflor (table 6.5.3.5-5). After normalisation with respect to bodyweight, relative liver weights were increased in WT (25%) and hPXR/hCAR (12%) mice but not PXRKO/CAR KO animals following exposure to sulfoxaflor.

Table 4.10.3.1.Study 5.4 (DAR Table 6.5.3.5-5): Mean terminal body and liver absolute and relative weights (n = 10)						
parameter	**WT**		**PXRKO/CARKO**		**hPXR/hCAR**	
	0	**750ppm**	**0**	**750ppm**	**0**	**750ppm**
Body weight (g)	22.4±1.0	22.2±1.8	24.1±1.3	24.7±1.4	25.5±1.4	24.8±1.5
Liver wt. (g)	0.99 ± 0.13	1.22 ± 0.19*	1.13 ± 0.06	1.21 ± 0.13	1.23 ± 0.10	1.35 ± 0.11
Rel. liver wt. (g/100g)	4.40 ± 0.54	5.50 ± 0.72*	4.69 ± 0.14	4.89 ± 0.39	4.84 ± 0.29	5.42 ± 0.28*

Values represent mean ± sd. A Student's t-test (2-sided) was performed on the results; *statistically different from control p<0.01.

Microscopic pathology:

Dietary administration of 750ppm sulfoxaflor in WT mice resulted in very slight or slight hypertrophy of centrilobular/midzonal hepatocytes (table 6.5.3.5-6). The cytoplasm of the hypertrophied hepatocytes in general was very slightly more eosinophilic than that of the wild type controls. Hepatocyte hypertrophy with increased cytoplasmic eosinophilia is consistent with an increase in smooth endoplasmic reticulum in the affected hepatocytes. The treatment-related hepatocyte hypertrophy correlated with increased liver weight in WT mice. In addition, there was a very slight treatment-related increase in hepatocellular mitosis in sulfoxaflor treated wild type mice as compared to its controls which was consistent with the increased BrdU labeling index in sulfoxaflor treated wild type mice (described later).

Table 4.10.3.1.Study 5.5 (DAR Table 6.5.3.5-6): Treatment-related histopathological changes in the liver (n=10)							
Diagnoses		**WT**		**PXRKO/CARKO**		**hPXR/hCAR**	
		0	**750ppm**	**0**	**750ppm**	**0**	**750ppm**
Hypertrophy; with altered tinctorial properties; hepatocyte; centrilobular/midzonal	*Very Slight*	0	**2**	0	0	0	**3**
	Slight	0	**8**	0	0	0	**7**
Mitotic alteration; increased; hepatocyte	*Very Slight*	1	**7**	0	0	0	1

The liver of sulfoxaflor treated PXRKO/CARKO mice was histologically comparable to that of untreated PXRKO/CARKO mice. There were no treatment-related histopathological changes in PXRKO/CARKO mice consistent with the pivotal role of PXR/CAR in the pathogenesis of hepatocyte hypertrophy and proliferation induced by sulfoxaflor as seen with wild type mice in this and other studies.

Very slight or slight treatment-related centrilobular/midzonal hepatocyte hypertrophy was observed in hPXR/hCAR mice following administration of 750ppm sulfoxaflor, similar to that observed in sulfoxaflor treated WT mice. Treatment-related hepatocyte hypertrophy correlated with increased relative liver weight observed in the hPXR/hCAR mice. However, in contrast to the sulfoxaflor treated WT mice, there was no treatment-related increase in hepatocellular mitosis. This was consistent with the lack of increased BrdU labeling index in sulfoxaflor treated hPXR/hCAR mice. These results strongly support the conclusion that while murine PXR/CAR mediates sulfoxaflor induced hepatocyte hypertrophy and proliferation, humanised PXR/CAR mediates only hepatocyte hypertrophy and not hepatocellular proliferation consistent with similar observations of increased liver size in

humans exposed to the prototypical PXR/CAR agonist Phenobarbital and lack of replicative DNA synthesis in human hepatocytes.

A very slight increase in hepatocellular mitosis was observed in one sulfoxaflor treated hPXR/hCAR mouse which was interpreted to be a spontaneous change due to its isolated incidence similar to that observed in one WT control. All other histopathologic changes observed in the livers of sulfoxaflor treated WT, PXRKO/CARKO or hPXR/hCAR mice were also considered spontaneous alterations not associated with the exposure to sulfoxaflor.

Targeted gene expression, enzyme activity, hepatocyte proliferation and protein content

1. CAR and PXR associated events:

To investigate if sulfoxaflor exposure resulted in a gene expression pattern similar to that observed with phenobarbital exposure, *Cyp2b10* and *Cyp3a11* were measured by quantitative RT-PCR using specific primers and probes, with murine β-actin as the reference gene (table 6.5.3.5-7). Additionally, Cyp2b enzyme activity was evaluated by benzyloxyresorufin (BROD) and pentoxyresorufin (PROD) O-dealkylase activities while benzylquinoline debenzylation activity (BQ) was used for the quantification of mouse Cyp3a11 activity (table 6.5.3.5-8). *Cyp2b10* gene induction, considered to be the prototypical gene response following PB exposure through activation of the constitutive active/androstane nuclear receptor (CAR) and/or PXR (both receptors have overlapping binding sites for both *Cyp2b10* and *Cyp3a11* and both receptors can mediate preferential induction of either gene), was induced in WT and hPXR/hCAR mice, but by how much is unclear. In WT C57BL/6J mice hepatic *Cyp2b10* is not constitutively expressed (or its level is so low as to be below the threshold of detection for the assay). A fold change in response to sulfoxaflor treatment cannot therefore be calculated over control values, although it can be seen that there is a large difference from WT controls (mean Ct of 29.8 ± 0.5 *vs.* mean Ct > 35, respectively, *higher values imply less mRNA expression*). Ct values > 35 are indicative of mRNA levels below the limits of detection for the assay. In contrast, basal *Cyp2b10* mRNA was expressed in the hPXR/hCAR animals (which correlates well with historical data and may be related to a lack of repression of *Cyp2b10* by hPXR relative to mPXR), with a marginal increase in *Cyp2b10* mRNA observed following sulfoxaflor treatment (approximately 4-fold increase). *Cyp2b10* mRNA levels were undetectable in control PXRKO/CARKO animals and as expected, remained so upon exposure to sulfoxaflor. These data indicate that *Cyp2b10* transcription is markedly upregulated by sulfoxaflor in WT mice, less so with hPXR/hCAR mice (due to high basal backround levels of transcription in 0 treatment controls) and practically undetectable in PXRKO/CARKO mice.

Hepatic *Cyp3a11* is constitutively expressed in WT, hPXR/hCAR and PXRKO/CARKO mice; therefore results can be expressed as a relative fold change over control values. In WT mice mean Ct values for *Cyp3a11* are: 21.1 ± 0.6 in controls and 20.1 ± 0.4 with sulfoxaflor exposure. These represent an approximate 2-fold increase in expression for sulfoxaflor treatment over control. *Cyp3a11* transcription was induced to a greater extent in the treated hPXR/hCAR mice when compared with WT animals (3.4-fold increase of controls), whereas following treatment with sulfoxaflor in PXRKO/CARKO mice, a down regulation of *Cyp3a11* mRNA was observed (2.5-fold decrease of controls). These data indicate that *Cyp3a11* transcription is induced by sulfoxaflor in both WT and hPXR/hCAR mice.

Table 4.10.3.1.Study 5.6 (DAR Table 6.5.3.5-7): Targeted gene expression expressed as change in mean Ct value compared to control.

parameter	WT		PXRKO/CARKO		hPXR/hCAR	
	0	750ppm	0	750ppm	0	750ppm
Cyp2b10 Ct	>35	29.8 ± 0.5	>35	>35	33.6 ± 2.1	31.7 ± 1.8
Cyp2b10 ΔCt	15.9 ± 3.3	9.0 ± 3.3	16.6 ± 3.2	18.5 ± 0.6	14.3 ± 2.3	10.4 ± 1.8
fold change	ND	ND	ND	ND	1	3.9
Cyp3a11 Ct	21.1 ± 0.6	20.1 ± 0.4	21.3 ± 0.7	22.5 ± 0.6	20.2 ± 0.7	18.5 ± 0.4
Cyp3a11 ΔCt	2.33 ± 0.66	-1.16 ± 2.97	0.62 ± 0.55	1.07 ± 0.38	0.88 ± 0.94	-3.38 ± 1.94
fold change	1	2.1	1	-2.5	1	3.4

Values represent the average threshold cycle (Ct) and delta Ct (ΔCt) values (Ct values from the gene of interest minus Ct values for the internal control gene) for each mouse group (n=10). Murine β-actin was employed as the internal control. Ct values > 35 are indicative of mRNA levels below the limits of detection for the assay. ND = not determined.

2. Cytochrome P450 enzyme analysis:

Pentoxyresorufin-*O*-dependentylation (PROD) and benzyloxyresorufin-*O*-debenzylation (BROD), which are associated with CAR activation, were used as markers for cytochrome P450 2b10 activity (table 6.5.3.5-8). Both PROD and BROD liver enzyme activities and give a measure of Cyp2b enzyme induction, were likewise significantly elevated in this study. Administration of sulfoxaflor elicited marked increases in PROD and BROD activities in WT mice of approximately 33- and 36-fold respectively. Under the same treatment, marginal induction of PROD and BROD was observed in the hPXR/hCAR mice (approximate increases of 2- and 3-fold respectively). As expected, there was no induction of either PROD or BROD activity in the sulfoxaflor-treated PXR KO/CAR KO mice.

Table 4.10.3.1.Study 5.7 (DAR Table 6.5.3.5-8): Hepatic Cytochrome P450 enzyme activity and content with respect to treatment.

parameter	WT		PXRKO/CARKO		hPXR/hCAR	
	0	750ppm	0	750ppm	0	750ppm
PROD[1]	3.3 ± 0.5	110.0 ± 35.5* (↑) 33-fold	4.7 ± 2.1	4.8 ± 2.4 NC	11.2 ± 3.2	27.9 ± 8.2* (↑) 2.5-fold
BROD[1]	7.6 ± 2.4	274 ± 86.9* (↑) 36-fold	20.3 ± 15.9	33.3 ± 34.9 (↑) 1.7-fold	83.0± 19.6	241 ± 67* (↑) 2.9-fold
BQ[2]	1.8 ± 0.3	4.8 ± 1.7* (↑) 2.7-fold	3.2 ± 0.5	3.3 ± 1.0 NC	3.9 ± 0.9	8.1 ± 2.3* (↑) 2.1-fold
total P450[3]	0.45 ± 0.10	0.86 ± 0.10* (↑) 1.9-fold	0.51 ± 0.06	0.51 ± 0.16 NC	0.64 ± 0.06	0.90 ± 0.14* (↑) 1.4-fold

Values are mean ± sd. [1] pmols product/min/mg protein; [2]nmols product/min/mg protein; [3]nmols/mg protein. A Student's t-test (2-sided) was performed on the results; *statistically different from control with $p<0.001$; NC: no change.

Benzyloxyquinoline debenzylation (BQ) was used for the quantification of murine Cyp3a11 activity (table 6.5.3.5-8). Similar levels of BQ induction were observed in WT and hPXR/hCAR mice following exposure to sulfoxaflor (approximate 3- and 2-fold increases respectively). No change in BQ activity was observed in PXRKO/CARKO mice under the same treatment. Basal Cyp2b10 and Cyp3a11 enzyme activity was significantly higher in hPXR/hCAR mice than WT mice. Administration of sulfoxaflor also resulted in significant increases in total hepatic microsomal P450 content of 1.9-fold and 1.4-fold in WT and hPXR/hCAR mice respectively. No change was observed in the PXR KO/CAR KO mice. In summary, P450 catalytic activities are only induced in WT and hPXR/hCAR mice in response

to sulfoxafloe exposure.

3. Hepatocellular Proliferation:

All mouse liver and duodenum (positive control) sections were analysed for BrdU incorporation as a measure of cell proliferation. Sulfoxaflor (XDE-208) increased the hepatocellular labeling index (S-phase) in the WT mice by approximately 4-fold and had little effect on cell proliferation in the PXRKO/CARKO or hPXR/hCAR animals (figure 6.5.3.5-1, table 6.5.3.5-9).

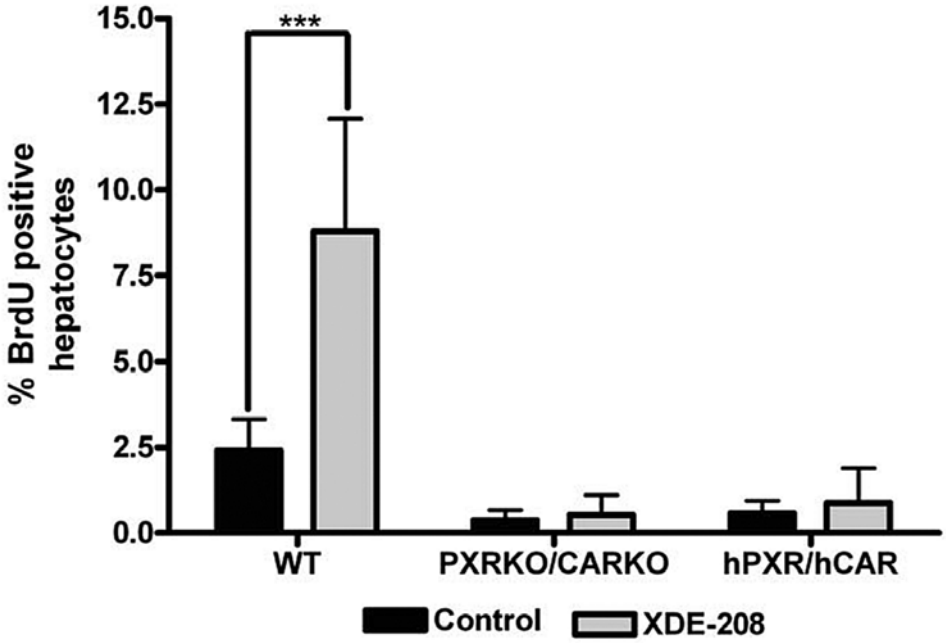


Figure 4.10.3.1.Study 5.1 (DAR Figure 6.5.3.5-1): Hepatic S-phase labeling indices in sulfoxaflor-treated mice. Data represents random sampling of 10 images per lobe (2) counting approximately 200,000 cells/animal group. Values are expressed as mean ± sd, n=10. A Student's t-test (2-sided) was performed on the results; ***statistically different from control p<0.001.

Table 4.10.3.1.Study 5.8 (DAR Table 6.5.3.5-9): Hepatic S-phase labeling indices in sulfoxaflor-treated mice (n=10)

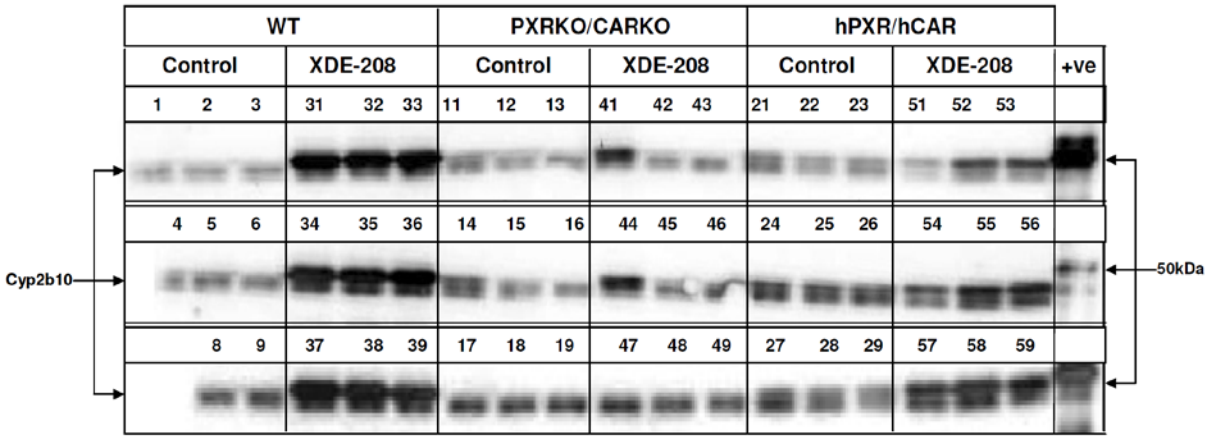
parameter	WT		PXRKO/CARKO		hPXR/hCAR	
	0	750ppm	0	750ppm	0	750ppm
% BrdU + hepatocytes	2.41±0.91	8.79±3.28*	0.38±0.29	0.53± 0.57	0.58±0.36	0.88±1.01
mean % change	100	365	100	139	100	152

Values represent mean ± sd. A Student's t-test (2-sided) was performed on the results; *statistically different from control p<0.001.

4. Qualitative protein analysis – Western blotting of hepatice microsomes:

SDS-PAGE was performed on samples from all animals to separate the microsomal proteins, including the cytochrome P450 enzymes and Western blotting was performed to identify specific changes in the level of *Cyp2b10* and *Cyp3a11* translated protein. Figure 6.5.3.5-2 clearly shows increased levels of Cyp2b10 and Cyp3a11 protein in response to sulfoxaflor treatment in both WT and hPXR/hCAR transgenic animals. In both cases, induction of protein is less for the hPXR/hCAR animals than the WT genotypes. No induction was observed in the PXR KO/CAR KO animals under any circumstance.

(A)



(B)

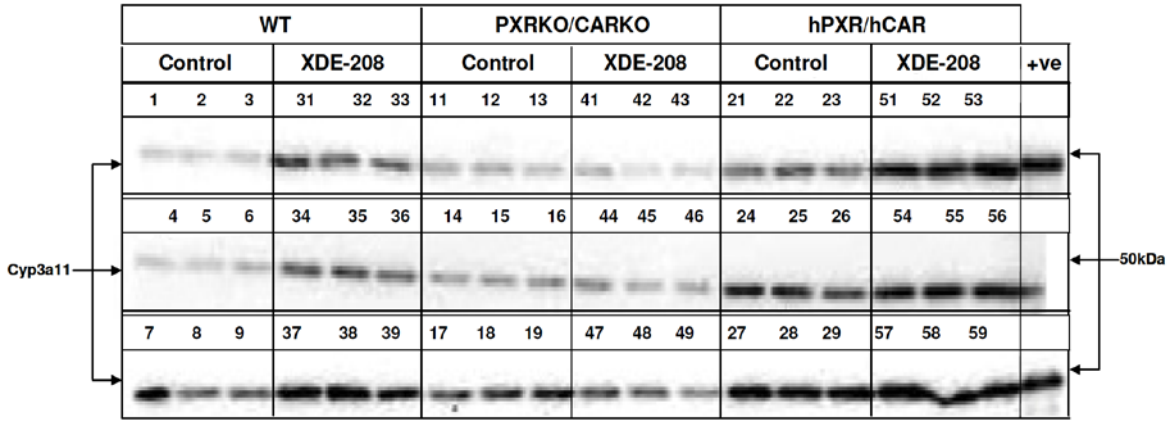


Figure 4.10.3.1.Study 5.2 (DAR Figure 6.5.3.5-2): Western blot of both (A) Cyp2b10 and (B) Cyp3a11 from microsomes isolated from each animal on the study. Lane number corresponds to each individual animal. For each sample, total protein of 1µg was loaded into each well of a 7.5% SDS page gel. Recombinant proteins were added as positive controls (Cyp3a11 was loaded at 0.01pmol. Cyp2b10 was loaded also at 0.01pmol).

Conclusions

Due to similarities in PB-like gene expression profiles and related endpoints (such as increased liver weight), it is likely that the hepatocyte and liver effects seen with exposure to sulfoxaflor are mediated through CAR/PXR nuclear receptor activation. The present study investigated: (1) CAR / PXR mediated sulfoxaflor-induced liver hypertrophy and hyperplasia in mice; and (2) whether the human receptors support these processes to a similar extent as the murine receptors. The mouse models employed were all based on a C57BL/6J

background strain.

The effects of sulfoxaflor were investigated in transgenic animals using double humanised PXR and CAR (hPXR/hCAR), double knockout PXR and CAR (PXRKO/CARKO), and wild-type (WT) C57BL/6J mice. In WT mice, sulfoxaflor at a dietary concentration of 750 ppm for 7 days caused increased liver weight, hepatocellular hypertrophy, and cell proliferation. Liver hypertrophy without hyperplasia was demonstrated in the hPXR/hCAR animals. Analysis of hepatocyte proliferation (S-phase) by BrdU immunohistochemistry confirmed that hyperplasia only occurred in the WT mice and not in the hPXR/hCAR or PXRKO/CARKO mice following sulfoxaflor treatment. Induction of the CAR and PXR target genes, *Cyp2b10* and *Cyp3a11*, was observed in both WT and hPXR/hCAR mouse lines following treatment with sulfoxaflor. In the PXRKO/CARKO mice, neither liver growth nor induction of *Cyp2b10* and *Cyp3a11* was seen following sulfoxaflor treatment, indicating that these effects are CAR/PXR dependent. These results strongly support the conclusion that while murine PXR/CAR mediates sulfoxaflor induced hepatocyte hypertrophy and proliferation, humanised PXR/CAR models mediate only hepatocyte hypertrophy and not hepatocellular proliferation consistent with similar observations of increased liver size in humans exposed to the prototypical PXR/CAR agonist phenobarbital (*Pirttiaho et al., 1982*) and lack of replicative DNA synthesis in human hepatocytes (*Hirose, et al., 2009*). Similarly, studies *in vitro* using primary hepatocyte cultures have shown that while rodents are sensitive to the proliferative effects, humans are not (*Lake, 2009*).

It is important to note that the hPXR/hCAR model used in this study contained the entire human CAR gene (exons 2 - 9) and that this construct was situated in place of the original murine CAR gene at the ATG start codon and operating under the native mouse promoter sequences. However a confounder in the analysis of CAR function across species is the discovery of splice variation in human CAR. The CAR2 and CAR3 splice variants together appear to account for perhaps up to one third of the total CAR transcript pool present in human hepatocytes and are predicted to encode functional receptor proteins (*Auerbach et al., 2003; Dekeyser et al., 2009*). Further, both CAR2 and CAR3 have the significant property of encoding ligand-activated receptors that are not constitutively active, in contrast to the wild-type receptor, CAR1 (*Auerbach et al., 2003; Dekeyser et al., 2009*). It is also important to understand that we cannot be totally certain that hCAR and hPXR when expressed in the mouse can function exactly as the genes do when they are expressed in human cells without much more indepth investigations that are beyond the scope of the present study.

Comparable with the pilot study in section B.6.5.3.4, (CXR0821), sulfoxaflor behaved in a similar manner to phenobarbital-mediated induction in WT mice. The mouse nongenotoxic hepatocarcinogens phenobarbital (PB) and chlordane induce hepatomegaly characterised by hypertrophy and hyperplasia. Increased cell proliferation is implicated in the mechanism of tumour induction (*Ross et al., 2010*). Phenobarbital like induction was demonstrated by a near 2-fold induction of total cytochrome P450 and markedly increased PROD and BROD activities (indicative of Cyp2b activity). This similarity to phenobarbital induction was confirmed by the increased expression of *Cyp2b10* mRNA demonstrated by RT-PCR and by immunoblots showing increases in Cyp2b10 protein. However, in the hPXR/hCAR animal model, under the same conditions, induction of *Cyp2b10* activity, protein and mRNA was markedly less than that observed in the WT animals following treatment with sulfoxaflor. As expected for a CAR/PXR dependent process, sulfoxaflor had no significant effect on *Cyp2b10* expression or catalytic activity in PXRKO/CARKO animals. Human *CYP3a4* and the murine homolog *Cyp3a11* are the major cytochrome P450 genes regulated by PXR. Sulfoxaflor-mediated *Cyp3a11* induction, as determined by BQ activity (Cyp3a enzymatic activity), RT-

PCR and immunoblotting was observed to a similar extent in the "humanised" and WT mice, but was not seen in the PXRKO/CARKO mice.

CAR-mediated hepatomegaly is a transient, adaptive response and augments the ability of the liver to clear an acute xenobiotic stress (*Huang et al., 2005*). In contrast, chronic CAR activation in rodents results in hepatocarcinogenesis (*Yamamoto et al 2004*). In both acute and chronic xenobiotic responses, hepatocyte DNA replication is increased and apoptosis is decreased. These effects are absent in CAR null mice, which are completely resistant to the tumourigenic effects of chronic xenobiotic stress. In the acute response, direct up-regulation of the oncogene *Mdm2* and its expression by CAR contributes to both increased DNA replication and inhibition of p53-mediated apoptosis (*Huang et al., 2005*).

The well-known differences in rodent and human xenobiotic responses raise the issue of the relevance of these rodent results to liver carcinogenesis in humans. Studies in hCAR mice indicate that CAR activators also increase DNA replication and inhibition of apoptosis (*Huang et al 2005*). It is unclear if this would result in the promotion of tumourigenesis in humans. Generally, most study authors agree that long-term barbituate treatment (a strong activator of CAR) is not associated with an increased incidence of liver tumours in humans (*Olsen et al 1995*) though there have been isolated reports suggesting otherwise (*Ferko et al., 2003; Vazquez et Marigil 1989*). In addition, prolonged administration of phenobarbital in human studies has been shown to increase liver size, which is associated with hepatocellular hypertrophy (*Aiges 1980*). Similar conclusions have been reached with fibrates and other nongenotoxic agents.

In summary, Sulfoxaflor exhibited greater activity towards the mouse CAR / PXR than the human CAR / PXR. The difference in hepatic response between wild type and humanised mice in this study is considered to be mediated via species specific features of CAR / PXR. The data shows that the human CAR / PXR construct supports sulfoxaflor-induced hypertrophy but not hyperplasia, a situation similarly seen with phenobarbital exposure in humans. The hyperplastic response is thought to be a major contributing factor in determining the potential for hepatocellular carcinogenesis in rodents. This study demonstrates that a significant species response is due to the CAR / PXR genotype present and questions the relevancy of sulfoxaflor-induced liver tumours in rodents with respect to liver tumour risk in humans where it may not act as a liver carcinogen.

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Study 6: Human Relevance Framework for Liver Tumours. DAR Section B.6.5.3.6.

When all of the data described in previous sections were used in a Human Relevance Framework (HRF) analysis, the conclusion was that the observed sulfoxaflor-induced rodent liver tumours occurred via a CAR-mediated MoA for which there is a high level of confidence. There is no evidence of increased hepatocellular proliferation in humans exposed to high doses of phenobarbital (PB), which operates by this MoA. Based on a previous MoA assessment, PB is considered not to be a hepatocarcinogen in humans (Lamminpaa et al., 2002; Olsen et al., 1995). Furthermore, a hepatocarcinogenic response in rodents for compounds which have data to support a PB-like MoA is considered not relevant to humans (US EPA, 2008). On this basis, the rodent liver tumours associated with administration of high dose levels of sulfoxaflor are thought to not pose a cancer hazard to humans.

Report: LeBaron, M.J., Rasoulpour, R.J., Geter, D.R., Billington, R. and Gollapudi,

B.B. (2010). XDE-208: Mode of action and human relevance framework analysis for XDE-208-induced rodent liver tumors. Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan, 48674

Report No.: Study ID: 100291. DECO HET DR-0404-3134-118

Dates: 2010

Guidelines: Not applicable. Not required for EU dossier submission.

GLP: Not applicable.

Deviations: None. This is an acceptable overview of all the data presented thus far in section B6.5 as pertains to sulfoxaflor-induced liver tumours in rodents and the toxicological relevancy of this effect to man.

Deficiencies: None. General discussion document.

Executive Summary: Sulfoxaflor causes liver tumours in rodent carcinogenicity studies via a proposed nuclear receptor-mediated mode-of-action (MoA) through the following key events:

(1) CAR receptor activation and;

(2) Increased hepatocellular proliferation, leading to

(3) Hepatocellular tumours.

These key events have been evaluated in a series of MoA studies aimed at examining the causality of sulfoxaflor's induction of liver tumours in the chronic studies. This document represents the weight of evidence approach used to evaluate the data based upon the Bradford-Hill criteria followed by subsequent application in a Human Relevance Framework (HRF). The conclusion from this evaluation is that the observed sulfoxaflor-induced rodent liver tumours occur via a CAR-mediated MoA for which there is a high level of confidence. Activation of rodent CAR (and minor contribution of PXR) produces a cascade of alterations in gene transcription that leads to increased hepatocellular proliferation, a critical event in the development of liver tumours, and similar to the established MoA for phenobarbital (PB). On the other hand, PB in humans results in activation of CAR and PXR leading to the induction of cytochrome P450 (CYP) enzymes; however, different enzymes are induced in humans compared to rodents and, more importantly, there is no evidence of increased hepatocellular proliferation in humans. Furthermore, extensive epidemiologic studies in humans exposed to levels of PB comparable to those in rodent bioassays did not find an increased risk of liver cancer. This finding was reinforced in the course of these studies with sulfoxaflor, where humanised CAR/PXR knock-in mice were refractory to the hepatocellular proliferative effect of sulfoxaflor, whereas wild-type mice demonstrated increased proliferation (section B6.5.3.5). Based on a previous MoA assessment, PB is not a hepatocarcinogen in humans. Furthermore, a hepatocarcinogenic response in rodents for compounds which have data to support a PB-like MoA is not relevant to humans. On this basis, the rodent liver tumours associated with administration of high dose levels of sulfoxaflor would not pose a cancer hazard to humans.

Introduction: Sulfoxaflor (XDE-208, X11422208, XR-208, [1-(6-Trifluormethylpyridin-3-yl)ethyl)](methyl)-oxido-l4-sulfanylidenecyanamide) is a compound with insecticidal properties mediated via its agonism at the highly abundant insect nicotinic acetylcholine receptor (nAChR). During the conduct of regulatory repeat-dose guideline rodent toxicology studies treatment-related liver effects were noted. Furthermore, in chronic/carcinogenicity dietary studies in mice and rats increased incidence of hepatocellular tumours (adenomas and carcinomas) was identified. In order to understand the basis for the sulfoxaflor-induced

rodent liver effects, several mode of action (MoA) investigations and studies were conducted. These studies for both the relevant repeat-dose toxicity studies and MoA studies can be found in the appropriate sections of the DAR. The synthesis of the relevant toxicity and MoA studies of sulfoxaflor herein provides the context from which to fully evaluate the proposed MoA. This analysis is based on the specific mechanistic data generated following exposure to sulfoxaflor and supports a strong link between constitutive androstane receptor (CAR)-activation and the associated phenotype. Furthermore, the experiments also rule-out involvement of other nuclear receptors (e.g., AhR, PPAR-α) and other potential contributors to rodent hepatocarcinogenesis such as genotoxicity and cytotoxicity. The MoA analysis is then followed by a human relevance framework analysis addressing the relevance of the rat developmental effects to humans.

Modes of action in rodents for chemically-mediated hepatocellular carcinogenesis:

Most hepatocarcinogens can be classified as to mode(s) of action for hepatocarcinogenesis through mechanistic studies and, based on this information; a rational evaluation can be made in terms of the relevance to humans (*Cohen, 2010*). Since the key events that lead to the development of cancer can be identified in rodents within 13 weeks of administration of the carcinogen, screening assays for carcinogenesis could be 13 weeks administration of the compound or less. Short-term assays can provide a detailed dose response and mechanistic basis for the effect in rodents and provide the basis for a rational extrapolation to possible human effects. Several modes of action have been identified for liver carcinogenesis in both humans and in rodent models and those applicable to the rodent model are listed in table 6.5.3.6-1.

Table 4.10.3.1.Study 6.1 (DAR Table 6.5.3.6-1): Modes of action for hepatocellular carcinogenesis.

point	mechanism of interest
I	**DNA reactivity**
II	Increased cell proliferation: *(a.) Receptor mediated:* i. PPAR (peroxisome proliferation) ii. enzyme induction (CAR, PXR, AHR) **iii. estrogen** iv. statins **v. cytotoxicity** vi. other *(b.) Non-receptor mediated:* **i. cytotoxicity** **ii. infections** **iii. iron (copper) overload** iv. increased apoptosis (e.g., fumonisin B1) v. other

Modes of action in **bold** are likely to be relevant to humans.

Postulated Key Events: The sequence of events in sulfoxaflor's proposed MoA for liver tumours includes:

- Activation of the constitutive androstane receptor **(Key Event #1)**
- Induction of liver CYP enzymes

- Increased liver weight and hepatic hypertrophy
- Increased hepatocellular proliferation **(Key Event #2)**
- Increased incidence of pre-neoplastic, altered hepatic foci
- Hepatocellular adenoma formation **(Key Event #3)**

(1) Key Event #1: Data supporting activation of the constitutive androstane receptor (CAR)

Induction of cytochrome P450 (CYP) enzyme activity is a well-known MoA for rodent hepatocarcinogenesis with phenobarbital (PB) as a standard example (*Whysner et al., 1996; Holsapple, et al., 2006*). PB is non-genotoxic and is considered a tumour promoter in rodents. The key events for this MoA include activation of CAR leading to CYP isozyme induction, increased hepatocellular proliferation, and subsequent induction of proliferative lesions in the liver including hepatocellular foci, adenomas, and carcinomas (*Cohen, 2010*). There is uncertainty as to whether the CYP induction is a critical step (necessary) or an indicator of chemical activity that is secondary to activation of CAR (associative). Activation of rodent CAR produces a cascade of alterations in gene transcription that leads to increased hepatocellular proliferation in rodents, a critical event in the development of liver tumours (*Whysner et al., 1996; Cohen, 2010*). In humans, PB results in activation of CAR and the pregnane X receptor (PXR) leading to the induction of CYP enzymes as in rodents; however, different enzymes are induced in humans compared to rodents (*Lambert et al., 2009*) and, more importantly, there is no evidence of increased hepatocellular proliferation in humans. Extensive human epidemiologic studies at PB exposure levels similar to those used in rodent bioassays do not result in increased cancer risks (*Whysner, et al., 1996; Lamminpaa et al., 2002*). Based on this assessment, PB is not a hepatocarcinogen in humans. Therefore, a non-genotoxic hepatocarcinogenic response in rodents due to a PB-like MoA is not relevant to humans (*Holsapple, et al., 2006*). The important role of CAR in PB-induced liver tumour formation in rodents has been demonstrated in genetically engineered mice lacking this nuclear receptor (reviewed in *Lake, 2009*). In CAR knockout (CARKO) mice, PB exposure does not induce Cyp2b forms or liver enzyme activity, increase liver weight, or stimulate replicative DNA synthesis. Also, no liver tumours were observed in CARKO mice following initiation with diethylnitrosamine (DEN) and promotion with PB.

Demonstration of CAR activation and the associated CYP gene and enzyme induction (i.e., Cyp2b10 in mice and Cyp2b1 in rats) are the first key events. Binding to CAR has been occasionally identified as an independent key event, but activation of CAR has been shown to occur by two independent mechanisms: 1) direct agonism by a ligand such as TCPOBOP (1,4-bis [2-(3,5-dichloropyridyloxy)]benzene), or 2) indirectly by compounds such as PB that activate CAR through a phosphorylation-dependent mechanism (*Rencurel et al., 2006*). Regardless of the means of CAR activation, the key events related to the MoA are identical. Therefore CAR activation, and not necessarily direct binding, is the most appropriate and measureable first key event.

Associative, supportive evidence that can aid in substantiation of CAR activation (and hence a CAR-mediated MoA) includes liver weight increases accompanied with microscopic hepatocellular hypertrophy, and this is commonly seen following exposure to PB-like compounds. PB-induced liver hypertrophy is normally observed in the centrilobular region of the liver lobule, although some related compounds may produce either a diffuse hypertrophy or hypertrophy in other regions of the liver lobule (*Lake, 2009*). Specifically, morphological features of enzyme induction in mice and rats can be characterised by light microscopy and/or

increased smooth endoplasmic reticulum (SER) by electron microscopy (EM). These changes are readily reversible upon discontinuance of administration of the chemical.

Several studies have been conducted with sulfoxaflor that support CAR (or possibly PXR) activation as a key event for liver tumour formation in rodents. These studies have focused on the CAR-dependent induction of genes responsible for some of the cytochrome P450 enzymes.

Evidence of Induction of CAR regulated cytochrome P_{450} and P_{450} isoenzymes

a) Gene expression analysis

TaqMan RT-PCR analysis (sections B6.5.3.2; *Geter and Card, 2010* and B6.5.3.3; *Geter et al., 2010*) was utilized in mice and rats to investigate (*i.e.*, rule-in or rule-out) nuclear receptor-mediated pathways related to the following: AhR signaling, CAR signaling, PXR signaling, and PPAR-α signaling.

In male and female mice, (table 6.5.3.6-2, original data taken from table 6.5.3.3-3) there were dose-related increases in levels of CAR mediated *Cyp2b10* transcript. After 7 days of treatment, there was a 42.1- and 54.8-fold difference in males versus the control group for the 500 and 750 ppm groups, respectively. For females, a 20- and 30.8-fold difference was seen in the 1000 and 1500 ppm groups, respectively. After 28 days, a 61.7-fold difference was seen at 300 ppm and after 90 days a 56.5-fold difference in male mice compared to controls. In females, a 93.9 fold difference was seen at 1500 ppm after 28 days and a 53.9-fold difference was seen at the same dose after 90 days.

Table 4.10.3.1.Study 6.2 (DAR Table 6.5.3.6-2): Targeted gene expression expressed as fold change compared to control.

		Male			Female		
study	**dose (ppm)**	***Cyp2b10***	***Cyp3a11***	***Cyp4a10***	***Cyp2b10***	***Cyp3a11***	***Cyp4a10***
7 day	0	1.0	1.0	1.0	1.0	1.0	1.0
	500	42.1*	1.6	1.2			
	750	54.8*	2.7*	1.0			
	1000				20.0*	4.0*	-5.6
	1500				30.8*	6.6*	-3.3
28 day	300	61.7*	1.5	-1.6			
	1500				93.9*	5.6*	-1.8
90 day	750	56.5*	2.8*	-3.6			
	1500				53.9*	3.4*	-2.3

* Data were statistically different from the control (alpha = 0.05) and fold-change > 1.5

There were also minor increases in PXR-mediated *Cyp3a11* transcript at the same treatment levels and durations in male and female mice. No changes were seen in *Cyp4a10* induction, indicating a lack of PPARα-mediated signaling in the liver.

In another study in mice (section B6.5.3.4; *Elcomb 2010*), there were increases in PROD and BROD activity, increased expression of *Cyp3a11* and *Cyp2b10* mRNA demonstrated by RT-PCR, and increases in Cyp3a11 and Cyp2b10 proteins as shown by Western blotting.

In rats, there were only minor inductions of CAR mediated *Cyp2b1* after 3 or 7 days at 100 ppm (see table 6.5.3.2-5, section B6.5.3.2; *Geter and Card, 2010*) in male and female rats. However, in the 750 ppm group, after 3 and 7 days, there was a 586- and 559-fold increase, respectively, in males and a 399- and 315-fold increase in females. At 1500 ppm, there were even greater inductions observed in both sexes at both time points.

Similarly (but quantitatively less), there was an increase in PXR mediated *Cyp3a11* in both sexes after 3 and 7 days at ≥750 ppm. Significant induction was also seen for other CAR (*Cyp2b2*) and PXR (*Cyp3a3*) related genes. There was a slight induction of the AhR-mediated *Cyp1a1* transcript (up to ~12-fold at 1500 ppm); however, the magnitude seen was not reflective of prototypical AhR agonists. Gene expression analysis on rats was not done for animals treated longer than 7 days (see table 6.5.3.2-5).

In a preliminary study (section B6.5.3.1; *Geter and Kan, 2008*), increased expression of CAR and PXR related genes was seen in mice, as well as increased hepatocellular proliferation in mice and rats.

b) Hepatic Metabolic Enzyme Activity

As a result of the gene expression data, which indicated a significant elevation of the CAR-mediated *Cyp2b* family (*Cyp2b10* in mice and *Cyp2b1* in rats), a functional analysis of the Cyp2b enzyme was done (sections B6.5.3.2; *Geter and Card, 2010* and B6.5.3.3; *Geter et al., 2010*) using 7-Pentoxy-Resorufin *O*-Deethylation (PROD) and 7-Benzyloxy-Resorufin-*O*-Deethylation (BROD) activity. Increased PROD and BROD enzyme activity is one of the primary alterations observed following a prototypical, PB-like, CAR activation (*Lubet et al., 1985*). There was a slight induction of AhR mediated *Cyp1a1* gene expression in rats; thus, ethoxyresorufin-*O*-deethylase (EROD) activity was also evaluated to measure the resultant enzyme activity.

In mice, statistically significant ($p < 0.05$) increases in PROD activity were seen in males at ≥ 500 ppm (3-4 fold) and females at ≥ 1000 ppm (5 fold) after 7 days (see table 6.5.3.3-4, section B6.5.3.3; *Geter et al., 2010*). Similar increases were seen in males after 28 and 90 days at 300ppm and 750 ppm, respectively. In females, significant increases were also seen at 1500 ppm after 28 and 90 days, but to a lesser extent. The increases correlated with *Cyp2b10* gene expression. Significant increases in BROD activity were also seen in male and female mice at the same doses. Increases were also observed in EROD activity in both sexes; however, these changes were minor.

In rats, statistically significant ($p < 0.05$) increases in PROD activity were seen in males at ≥ 750 ppm after 3 (8-11 fold) and 7 days (9-10 fold). In females, even greater increases in PROD activity were seen at the same doses after 3 (5-12 fold) and 7 days (13-16 fold). Similarly, significant increases were also seen in BROD activity in both sexes at the same doses, but to a lesser extent. Only minor increases were seen in EROD activity in both sexes (see table 6.5.3.2-6, section B6.5.3.2; *Geter and Card, 2010*).

c) Genetically Engineered Mouse Models

Different Dual CAR-PXR knockout (PXRKO/CARKO) mice and mice humanised for PXR and CAR (hPXR/hCAR) were used to determine if CAR or PXR mediate sulfoxaflor liver effects and if the human receptors support these processes to a similar extent as the murine receptors (section B6.5.3.5; *Ross, 2010*). There were treatment-related increases in absolute

and relative liver weights in wild type (WT) and humanised mice but not in the knockout animals. Absolute liver weights for WT and humanised mice in the 750 ppm sulfoxaflor groups were increased 24 and 9% of controls respectively, and relative liver weights were increased 25 and 12%, respectively. A minor increase of 7% was seen in knockout mice (table 6.5.3.6-3). Treatment related increased hepatocellular proliferation (365%) was seen in treated WT mice, but only minor changes in proliferation were seen in the humanised and knockout mice. Treatment-related hepatocyte hypertrophy was also observed in WT and humanised mice and increased mitotic figures were observed only in WT mice. No hepatocyte hypertrophy or increased mitotic figures were seen in knockout mice. In WT mice, a marked induction of total cytochrome P450, markedly increased PROD and BROD activities (Cyp2b selective substrates), increased expression of *Cyp2b10* mRNA (demonstrated by RT-PCR), and increases in Cyp2b10 protein (immunoblotting data) were observed after exposure to sulfoxaflor. However, in the humanised mice, induction of Cyp2b10 activity, protein and mRNA was markedly less than observed in the WT animals. In knockout animals, there were minimal effects on *Cyp2b10* expression or catalytic activity. Sulfoxaflor-mediated *Cyp3a11* induction, as determined by BQ activity (Cyp3a selective reaction), RT-PCR and immunoblotting was observed in the humanised and WT mice (to similar extents) but not in knockout mice.

Table 4.10.3.1.Study 6.3 (DAR Table 6.5.3.6-3): Summary of treatment-related (750 ppm) liver effects with CAR-PXR knockout mice and mice humanised for PXR and CAR (data summarised from section B6.5.3.5).

Liver parameter	Wild type	Humanized	Knockout
↑ Weight			
- Absolute (g)	↑24%	↑9%	↑7%
- Relative (g/100g)	↑25%	↑12%	↑4%
Hypertrophy	100%	100%	-
Mitosis	70%	10%*	-
Proliferation (% S-phase)	↑265%	↑52%	↑39%
Total P450	↑91%	↑41%	-
PROD (*Cyp2b10 via* CAR)	↑3245%	↑149%	↑1%
BROD (*Cyp2b10 via* CAR)	↑3515%	↑191%	↑64%
BQ (*Cyp3a11 via* PXR)	↑170%	↑109%	↑2%
Cyp2b10 expression (CAR)			
- RT-PCR	N/A	↑3.9x	N/A
- Immunoblots	↑↑↑	↑	-
Cyp3a11 expression (PXR)			
- RT-PCR	↑2.1x	↑3.4x	(↓2.5x)
- Immunoblots	↑	↑↑	-

N/A - not constitutively expressed; a fold change in response to sulfoxaflor treatment cannot be calculated over control values. ; – effect not observed. * Same incidence (1/10) seen in WT controls.↑↑↑ (large increase, ↑↑ moderate, ↑ (marginal)

d) Increased liver weight and hepatic hypertrophy

Although not direct evidence of causality of CAR mediated hepatic effects, increased liver weight and hypertrophy were observed in several repeat dose studies in mice and rats and are representative of the treatment-related effects that result from sulfoxaflor dietary administration. In male mice, statistically significant increases in absolute liver weight were seen at 3500 ppm after 28 days (↑ >92%) and at ≥ 750 ppm after 90 days of treatment (↑21 – 75%). Significant increases were also seen in relative liver weights at 750 ppm after 7 days

(↑17%), at ≥ 1500 ppm after 28 days (↑30 – 93%), and at≥ 750ppm after 90 days (↑25 – 83%). Relative liver weights were also significantly increased in WT and humanised mice, while knockout mice had liver weights similar to the respective controls. Significant increases in absolute liver weight were seen in WT mice. Additionally, increased incidences of hepatocellular hypertrophy were seen at high doses after 7 (750 ppm), 28 (≥1500 ppm), and 90 days (≥ 750 ppm) of treatment. The severity also increased with dose and duration (table 6.5.3.6-4).

Table 4.10.3.1.Study 6.4 (DAR Table 6.5.3.6-4): Summary of microscopic hepatocellular hypertrophy in liver of male CD-1 mice.

Dose	Very slight	Slight	Moderate
7-day treatment:			
0	0/5	0/5	0/5
500 ppm	0/5	0/5	0/5
750 ppm	**5/5**	0/5	0/5
28-day treatment:			
0	0/5	0/5	0/5
300 ppm	0/5	0/5	0/5
1500 ppm	0/5	**5/5**	0/5
3500 ppm	0/5	0/5	**5/5**
90-day treatment:			
0	9/10	0/10	0/10
100 ppm	9/10	0/10	0/10
750 ppm	0/10	**9/10**	**1/10**
1250 ppm	0/10	**1/10**	**9/10**

Data are number of mice with the observation/number of mice examined. **Bold** indicates treatment-related.

In male rats, statistically significant increases were only seen in relative liver weight after 3 days (1500 ppm,↑14%) and 7 days (≥750 ppm, ↑11 – 23%) of treatment. After 28 days, significant increases in absolute (↑26 – 45%) and relative (↑29 – 59%) liver weights were seen at ≥ 1000 ppm. After 90 days, increases absolute liver weight was seen at 1500 ppm (↑ >25%) and increased relative weight at≥ 750 ppm (↑14 – 41%). Additionally, an increased incidence of hepatocellular hypertrophy was seen at≥ 100 0 ppm after 28 days and≥ 750 ppm after 90 days of treatment. Similar to mice, severity increased with dose and duration (table 6.5.3.6-5; after 90 days of treatment with 1500 ppm sulfoxaflor, 9 of 10 animals had moderately graded hypertrophy and one animal was graded slight). Male rats treated for 90 days with a 28 day recovery period had liver weights comparable to control animals and demonstrated reversibility of the microscopic hepatocellular hypertrophy after removal of the test compound and administration of control diet (following 28 days of recovery only 2 of 10 animals had very slight hypertrophy and the others were considered to have reverted to a normal histological appearance).

Table 4.10.3.1.Study 6.5 (DAR Table 6.5.3.6-5): Summary of microscopic hepatocellular hypertrophy in liver of male F344 Rats.			
Dose	**Very slight**	**Slight**	**Moderate**
28-day treatment:			
0	0/5	0/5	0/5
300 ppm	0/5	0/5	0/5
1000 ppm	0/5	**2/5**	**3/5**
2000 ppm	0/5	0/5	**5/5**
90-day treatment:			
0	0/10	0/10	0/10
100 ppm	0/10	0/10	0/10
750 ppm	0/10	**7/10**	**3/10**
1500 ppm	0/10	**1/10**	**9/10**
90-day treatment with 28 day recovery:			
0	0/10	0/10	0/10
1500 ppm	2/10	0/10	0/10

Data are number of rats with the observation/number of rats examined. **Bold** indicates treatment-related.

(2) Key Event #2: Data supporting increased hepatocellular proliferation

The second key event is a demonstration of an increase in proliferation of hepatocytes. Typically, for most compounds including PB, the increase in labeling index appears very quickly, usually within one to two weeks of treatment initiation and the index returns to normal by four weeks of administration; however, a PB-induced increase in hepatocellular proliferation in mice was more prolonged than in rats (*Kolaja et al., 1996a*). Although the hepatocyte-labeling index returns to control levels even with sustained PB treatment, the livers of treated animals are enlarged and stereologic studies indicate that hepatocellular proliferation is still enhanced due to the increase in the total number of hepatocytes per animal (*Lake, 2009*).

Increased hepatocellular proliferation is a key event for a PB-like MoA for hepatocellular carcinogens. The increased hepatocellular proliferation leads to the induction of proliferative lesions in the liver, including foci, adenomas, and carcinomas (*Cohen, 2010*). The progression from foci of altered cells (preneoplastic foci) to hepatocellular adenomas/carcinomas following PB administration is well documented in rodents for PB (*Whysner et al., 1996*). PB administration results in a dose dependent increase in cell proliferation within foci that is associated with the ability of foci to progress to hepatocellular adenomas (*Klaunig, 1993*). Although development of altered hepatic foci is sometimes listed as a key event for PB-like MoAs, the foci are a reflection of the hepatocellular proliferation which is the actual key event that leads to selective clonal expansion resulting in the formation of microscopic hepatocellular foci and the subsequent development of adenomas and/or carcinomas. The recent publication by *Cohen* (2010) does not include hepatocellular foci as a specific key event in the data necessary to support a CAR-mediated MoA.

Inhibition of apoptosis, which is sometimes listed as a separate key event for a CAR-mediated/PB-like MoA, primarily pertains to the tumourigenic progression of preneoplastic hepatocytes within foci of altered cells rather than other liver tissue (non-focal hepatocytes) (*Schulte-Hermann et al., 1989, 1990; Kolaja et al., 1996b; Whysner et al., 1996; Lake, 2009*). The data for inhibition of apoptosis in foci of altered cells are primarily derived from initiation-promotion experiments rather than from studies with promoters such as PB alone. PB promotes growth of foci by inhibition of cell loss due to apoptosis and to phenotypic remodeling (*Schulte-Hermann et al., 1990*). The frequency of apoptosis in foci is enhanced after PB withdrawal. Although *Goldworthy and Fransson-Steen* (2002) did identify occasional extrafocal (i.e., not within foci of altered cells) apoptotic bodies in mice treated

with DEN and/or PB, these apoptotic bodies were limited to the adjacent area surrounding foci of altered cells. Thus, there should be no expectation that standard regulatory toxicity studies in mice would detect PB-like inhibition of apoptosis unless a specific analysis of foci, which typically develop relatively late in long-term studies, were conducted. Also, shorter term mechanistic studies in mice with PB or PB-like compounds typically do not develop hepatocellular foci for months (*Goldsworthy and Fransson-Steen, 2002*). Studies in mice initiated with DEN followed by promotion with PB suggest that cell proliferation has a major role in foci growth and that inhibition of apoptosis is only a minor determinant of tumour promotion (*Goldsworthy and Fransson-Steen, 2002; Bursch et al., 2005b*). The recent publication by *Cohen* (2010) does not include inhibition of apoptosis as a specific key event in the data necessary to support a CAR-mediated MoA.

Evidence of Proliferation

a) Nuclear label incorporation

Hepatocellular proliferation data are available for mice and rats (3 days to 90 days). Immunohistochemical staining for BrdU- or Ki-67-labeled nuclei as a measure of hepatocellular proliferation was based on interpretation of the nuclear staining intensity and an approximation of the location within the three hepatolobular zones. In mice, statistically significant increases in hepatocellular proliferation (BrdU) were seen in males after 7 days at ≥ 500 ppm (centrilobular region) and 750 ppm (midzonal region). No increases were seen in the periportal region (table 6.5.3.6-6). Additionally, no treatment related increases in proliferation were seen after 28 and 90 days. In female mice, significant increases in proliferation were seen in all regions at≥ 500 ppm at 7 days. No treatment related increases were seen after 28 and 90 days.

Table 4.10.3.1.Study 6.6 (DAR Table 6.5.3.6-6): Summary of hepatocellular proliferation in liver lobules of CD-1 mice.

Dose	CL	MZ	PP
7-day treatment (BrdU):			
0	1.0	1.0	1.0
500 ppm	**2.68**	**3.37**	1.41
750 ppm	**3.18**	**3.86**	1.45
28-day treatment (Ki67):			
0	1.0	1.0	1.0
300 ppm	-1.10	1.17	1.37
90-day treatment (Ki-67):			
0	1.0	1.0	1.0
750 ppm	-2.84	-1.09	-1.02
7-day treatment (BrdU) - females:			
0	1.0	1.0	1.0
500 ppm	**4.12**	**3.58**	**2.70**
750 ppm	**4.88**	**4.66**	**3.78**

Data are relative fold-change compared to control values. CL = centrilobular, MZ = midzonal, PP = periportal. **Bold** indicates treatment-related.

These proliferation data were generated by evaluation of BrdU-labeled hepatocytes, following 7 days of osmotic pump infusion before tissue collection. Consistent with the known proliferative profile of PB (reviewed in *Cohen, 2010*), prolonged administration (28 to 90 days) of tumourigenic concentrations of sulfoxaflor resulted in a return to “normal” levels of hepatocellular proliferation. In the case of PB, the livers of treated animals were enlarged; however, stereologic studies indicate that hepatocellular proliferation is still enhanced compared to control animals due to the increase in the total number of hepatocytes per animal

(*Lake, 2009*).

In rats, statistically significant increases as measured by Ki-67 staining were seen in males at tumourigenic doses ≥ 750ppm in the centrilobular and midzonal regions after 7 days. Similar to the response observed in mice, administration of sulfoxaflor for 28-days did not result in sustained hepatocellular proliferation, even at 2000 ppm. (table 6.5.3.6-7). In female rats, significant increases were seen at 1500 ppm after 7 days and 2000 ppm after 28 days in the centrilobular region only. No treatment related increases were seen in males or females after only 3 days.

Table 4.10.3.1.Study 6.7 (DAR Table 6.5.3.6-7): Summary of hepatocellular proliferation in liver lobules of F344 rats.

Dose	CL	MZ	PP
3-day treatment (Ki-67) - males:			
0	1.0	1.0	1.0
100 ppm	1.32	1.40	1.39
750 ppm	1.49	1.20	1.11
1500 ppm	1.10	1.05	-1.16
7-day treatment (Ki67) - males:			
0	1.0	1.0	1.0
100 ppm	1.23	1.57	1.29
750 ppm	**2.33**	**1.94**	**2.16**
1500 ppm	**3.38**	**2.42**	**2.88**
28-day treatment (Ki-67) - males:			
0	1.0	1.0	1.0
2000 ppm	**1.69**	-1.55	1.08
7-day treatment (Ki-67) - females:			
0	1.0	1.0	1.0
100 ppm	0.75	0.79	0.96
750 ppm	1.51	1.04	1.24
1500 ppm	**2.66**	1.37	1.57
28-day treatment (Ki-67) - females:			
0	1.0	1.0	1.0
2000 ppm	**3.03**	0.84	0.87

Data are relative fold-change compared to control values. CL = centrilobular, MZ = midzonal, PP = periportal. **Bold** indicates treatment-related.

Evaluation of hepatocellular proliferation data from mice and rats over a range of study durations (3 to 90 days) and dietary concentrations of sulfoxaflor (100 to 2000 ppm) are informative for the second key event in rodent liver tumour induction associated with CAR activation. In addition, specificity of CAR-mediated, sulfoxaflor-induced hepatocellular proliferation in rodent liver was shown by experiments using CARKO/PXRKO and hCAR/hPXR mice (section B6.5.3.6). Specifically, in wildtype C57BL/6J mice, treatment with sulfoxaflor at 750ppm in the diet for 7 days induced a clear, statistically significant 3.65-fold induction in the number of proliferating hepatocytes. This result was consistent with previous studies in rats and in the CD-1strain of mice used in the combined chronic/carcinogenicity study, subchronic studies, and other MoA studies. Importantly, neither the CARKO/PXRKO or hCAR/hPXR mice had statistically or biologically increased hepatocellular proliferation (1.39- or 1.52-fold, respectively), underscoring the difference in rodent and human responses to CAR activation, and the qualitative differences in nuclear receptor-mediated hepatic responses (table 6.5.3.6-8).

Table 4.10.3.1.Study 6.8 (DAR Table 6.5.3.6-8): Hepatic S-phase labeling indices in 7-day sulfoxaflor-treated mice (n=10)

parameter	WT		PXRKO/CARKO		hPXR/hCAR	
	0	750ppm	0	750ppm	0	750ppm
% BrdU + hepatocytes	2.41±0.91	8.79±3.28*	0.38±0.29	0.53± 0.57	0.58±0.36	0.88±1.01
mean % change	100	365	100	139	100	152

Values represent mean ± sd. A Student's t-test (2-sided) was performed on the results; *statistically different from control p<0.001.

In addition, in the 28-day study in mice there was also a very slight or slight treatment related increase in the overall numbers of hepatocytes in mitosis in the animals treated with 1500 or 3500 ppm sulfoxaflor when compared to control animals, *Thomas et al., 2008.* XR-208: 4-Week Repeated Dose Dietary Toxicity Study in Crl: CD1(ICR) Mice). This is consistent with the general dose-related increase in hepatocellular proliferation noted in the molecular (BrdU and Ki-67) MoA analyses.

b) Pre-neoplastic and altered hepatic foci

Increased hepatocellular proliferation is a key event for a PB-like MoA for nuclear receptor-mediated hepatocellular carcinogens. The increased hepatocellular proliferation leads to the induction of proliferative lesions within the liver, including foci, adenomas, and carcinomas (*Cohen, 2010*). Sulfoxaflor exposure resulted in an increased incidence of hepatocellular foci (eosinophilic and clear cells) in male mice at 750 ppm but not at non-hepatotumourigenic dietary concentrations of 25ppm or 100ppm. (table 6.5.3.6-9).

Table 4.10.3.1.Study 6.9 (DAR Table 6.5.3.6-9). Total Incidences of hepatocellular foci in CD-1 mice treated with sulfoxaflor for 18 Months.

Males: Dose ppm	0	25	100	750
# Mice with basophilic foci, focal or multifocal	3	1	4	2
# Mice with eosinophilic foci, focal or multifocal	3	2	3	**10**
# Mice with vacuolated or clear foci, focal or multifocal	1	0	1	**6**

Females: Dose ppm	**0**	**25**	**250**	**1250**
# Mice with basophilic foci, focal or multifocal	0	0	1	0
# Mice with eosinophilic foci, focal or multifocal	0	0	0	0
# Mice with vacuolated or clear foci, focal or multifocal	0	0	0	0

Data extracted from study report 081102; *Thomas et al., 2010,* summarised in section B.6.5.2.1. Data are the number of animals with the specified observation (total number of animals is 50 per dose).

Table 4.10.3.1.Study 6.10 (DAR Table 6.5.3.6-10.) Incidence of hepatocellular foci in F344 rats treated with sulfoxaflor for 2 years				
Males: Dose ppm	**0**	**25**	**100**	**500**
Basophilic foci, focal or multifocal				
instances of 1 – 5	17	15	13	18
instances of 6 – 10	22	14	14	23
instances of 11 – 20	5	12	11	1
instances of 21 or more	0	1	1	0
total (all instances)	44	42	39	42
Eosinophilic foci, focal or multifocal				
instances of 1 – 5	26	33	26	20
instances of 6 – 10	7	2	7	11
instances of 11 – 20	2	0	3	7
instances of 21 or more	0	0	0	1
total (all instances)	35	35	36	**39**
Vacuolated or clear foci, focal or multifocal				
instances of 1 – 5	17	21	14	17
instances of 6 – 10	0	0	1	0
	17	21	15	17
Females: Dose ppm	**0**	**25**	**100**	**750**
Basophilic foci, focal or multifocal				
instances of 1 – 5	2	4	3	**9**
instances of 6 – 10	7	3	7	**17**
instances of 11 – 20	19	21	18	18
instances of 21 or more	19	18	18	**2**
total (all instances)	47	46	46	46
Eosinophilic foci, focal or multifocal				
instances of 1 – 5	26	32	30	36
instances of 6 – 10	0	0	1	2
instances of 11 – 20	0	1	0	0
instances of 21 or more	0	0	0	0
total (all instances)	26	33	31	**38**
Vacuolated or clear foci, focal or multifocal				
instances of 1 – 5	14	8	12	10
instances of 6 – 10	0	0	0	0
	14	8	12	10

Data extracted from study report 071187; *Stebbins et al., 2010*, summarised in section B.6.5.1.1. Data are the number of animals with the specified observation (total number of animals is 50 per dose).

Minor increases in hepatocellular foci were seen in rats of both sexes at the highest dose (500ppm / 750ppm). A very subtle dose effect is seen with the raw data from the original histopathology reports that is lost if reviewers only take note of the total instances of foci (table 6.5.3.6-10). Males show small increases in eosinophilic foci with groupings of 6 – 10 and 11 – 20 while females show larger numbers of basophilic foci with with groupings of 1 – 5 and 6 – 10. Although development of altered hepatic foci is sometimes listed as a key event for PB-like MoAs, the foci are a reflection of the hepatocellular proliferation which is the actual key event that leads to selective clonal expansion resulting in the formation of

microscopic hepatocellular foci and the subsequent development of adenomas and/or carcinomas. The recent publication by *Cohen* (2010) does not include hepatocellular foci as a specific key event in the data necessary to support a CAR-mediated MoA.

(3) Apical Endpoint/Key Event #3: Hepatocellular tumours

A large number of chemicals have been shown to induce rodent liver tumours (*reviewed in Lake, 2009, and references therein*). While the biological effect (i.e., hepatic adenomas and/or carcinomas) is equivalent, detailed mechanistic investigations have established genotoxic and nongenotoxic modes of action that can be applied to the pathogenesis of hepatocellular tumours. *Cohen* (2010) describes each of the known contributors, including the MoA for CAR-mediated effects, relevant for sulfoxaflor and detailed in this document.

Dietary administration of sulfoxaflor to CD-1 mice resulted in increased tumours of the liver in a dose dependent manner. Male mice were more susceptible to the development of adenomas and carcinomas than female mice. Treatment-related neoplastic effects consisted of statistically significant ($p < 0.01$) increases in the incidences of hepatocellular adenomas and carcinomas in high dose males and females when compared to their respective controls (see tables 6.5.2.1-8a and 6.5.2.1-8b in section B.6.5.2.1). Male mice were much more sensitive to the neoplastic effects of sulfoxaflor – 60% of the high dose males developed hepatocellular adenomas and/or carcinomas (*vs* 26% in male controls), as opposed to 11% of the high dose females (*vs* 2% in female controls).

In rats, statistically significant trends ($p<0.01$) were seen for both hepatocellular adenomas and the combined (adenomas/carcinomas). When compared to controls, a statistically significant increase in pairwise comparison was seen for hepatocellular adenomas ($p<0.01$) and combined adenomas/carcinomas ($p<0.05$, driven by the adenoma response) at the highest dose (500 ppm, 21.3mg/kg bw/d). The incidences of liver tumours at the high dose (33%) exceeded the testing laboratories historical control range of 2 – 12% for the adenomas or 2 – 14% for the combined liver tumours (see table 6.5.1.1-11 in section B.6.5.1.1).

Summary of sulfoxaflor liver tumour MoA:

The MoA for sulfoxaflor-induced rodent liver tumours is comparable to the MoA for PB-induced rodent liver tumours. The relevant molecular and pathological endpoints for sulfoxaflor-induced liver effects in mice and rats are summarised in tables 6.5.3.6-11 and 6.5.3.6-12, respectively. This analysis is based on the mechanistic and standard, repeat-dose toxicity studies in mice and rats administered sulfoxaflor. The table is organised such that the metrics are consistent with the established key events of nuclear receptor-mediated rodent hepatocarcinogenesis (*Lake, 2009; Cohen, 2010*).

Key event #1 for the sulfoxaflor-induced liver tumour MoA is defined as activation of the nuclear receptor (i.e., CAR). The activation of CAR by sulfoxaflor has not been investigated directly; however, a diagnostic, surrogate response of CAR activation is commonly associated with the induction of hepatic xenobiotic metabolising enzymes, i.e., cytochrome p450s, primarily in the Cyp2b family (*Ueda et al., 2002*). As previously mentioned, CAR activation can occur by either a direct (e.g., TCPOBOP) or indirect (e.g., PB) mechanism (*Rencurel et al., 2006*). Furthermore, PB has been shown to activate the PXR-inducible Cyp3a enzyme family, which is also seen following exposure to sulfoxaflor.

Table 4.10.3.1.Study 6.11 (DAR Table 6.5.3.6-11). Sulfoxaflor: temporality and dose

response for MoA key events related to CD-1 male mouse liver tumours at dose levels used for cancer studies.

Temporal →

Dose ↓

Dose (ppm)	Key Event 1		Key Event 2	
	CAR Receptor Activation Cyp2b10 Transcripts & Protein	Associated Event: Increased Liver Weights/ Hypertrophy	Hepatocellular Proliferation	Increased Hepatocellular Tumors
	7-90 Days	7-90 Days	7 Days	18 Months
25				-
100		-		-
300	+	-		
500	+	-	+	
750	+	+	+	+
≥1250*		+		
750 KO	-	-	-	
750 HU	+	+	-	

+ Indicates effect present, - indicates effect absent at indicated duration of treatment. Blank cell = no data. * Includes similar results from 1500 ppm and 3500 ppm. Abbreviations: KO – CAR/PXR knock-out mouse model, HU – CAR/PXR humanized knock-in mouse model.

Additional experiments performed with genetically engineered mouse models (previously mentioned and described further below) provide clear evidence of sulfoxaflor-mediated activation of hepatic CAR and PXR; specifically, CARKO/PXRKO-null mice did not respond to sulfoxaflor administration. Gene expression analysis was utilised to investigate (i.e., rule-in or rule-out) nuclear receptor-mediated pathways related to the following: AhR, CAR, PXR, and PPAR-α signaling. In mice exposed to a tumourigenic dose of 750 ppm, liver gene expression analysis indicated a clear, CAR-mediated response to sulfoxaflor, with contributions from PXR. Similar CAR activation was noted at 500 ppm after 7 days and 300 ppm after 28 days. Liver enzyme activity (7-Pentoxy-Resorufin *O*-Deethylation, PROD) at those same dietary concentrations supported the results of the gene expression analysis and was consistent with CAR activation. In CARKO/PXRKO animals treated with the same carcinogenic dietary concentration of sulfoxaflor (750 ppm), no evidence of CAR (or PXR) activation was noted, either by gene expression or liver enzyme activity. In animals with hCAR/hPXR, sulfoxaflor-treatment induced quantitatively less CAR activation compared to wild type mice; however, the PXR-associated response in hCAR/hPXR mice was equal to or greater than the induction noted in wild type mice.

Table 4.10.3.1.Study 6.12 (DAR Table 6.5.3.6-12). Sulfoxaflor: temporality dose response and reversibility for MoA key events related to F344 male rat liver tumours at dose levels used for cancer studies.

Temporal →

Dose ↓

Dose (ppm)	Key Event 1		Key Event 2		
	CAR Receptor Activation Cyp2b10 Transcripts & Protein	Associated Increased Liver Weights/ Hypertrophy	Hepatocellular Proliferation	Associated Liver Hypertrophy After Recovery	Increased Hepatocellular Tumors
	3-7 Days	7-90 Days	7 Days	90 Days Plus 28 Days Recovery	2 Years
25					-
100	-	-	-		-
500					+
750	+	+	+		
1000		+			
1500	+	+	+	-	
2000		+			

+ Indicates effect present, - indicates effect absent at indicated duration of treatment. Blank cell = no data.

As in mice, rats showed a similar CAR-mediated response after dietary administration of sulfoxaflor. Specifically, a dose-responsive increase in *Cyp2b1* transcript and associated enzyme (PROD/BROD) activity was identified. At dietary concentrations equivalent to a non-hepatotumourigenic dose (100 ppm), no significant induction of CAR-mediated, Cyp2b-associated response was observed; however, at doses at or above 750 ppm, clear induction of CAR-mediated transcription was seen. This response occurred at concentrations only slightly higher than the rat liver carcinogenic dietary concentration of 500 ppm. Furthermore, specificity for sulfoxaflor-induced CAR activation was illustrated by a further increase in *Cyp2b1* transcript levels in rats treated with 1500 ppm sulfoxaflor. In summary, the data for sulfoxaflor-induced, liver-specific, CAR-mediated effects in both mice and rats were consistent with the response seen with other CAR-inducers, e.g., PB, and are consistent with the first key event in CAR-mediated liver tumourigenesis.

Although not direct evidence of causality of CAR-mediated hepatic effects, liver weight increases accompanied with microscopic hepatocellular hypertrophy can provide associative support of a CAR-mediated MoA and is commonly seen following exposure to PB-like compounds. In mice exposed to non-tumourigenic concentrations of sulfoxaflor, no increase in liver weight or histopathological identification of centrilobular hypertrophy were identified. Conversely, when sulfoxaflor was administered at and above dietary concentrations that caused liver tumours≥(750 ppm), a clear dose -responsive increase in liver weight and hypertrophy were identified. These hepatic hypertrophic effects were also seen in the hCAR/hPXR mice, but not in the CARKO/PXRKO mice, further supporting the molecular and phenotypic basis for CAR-mediated, liver-specific effects of sulfoxaflor.

In rats, similar to what was seen with mice, no hypertrophic effects were noted in animals treated with less than a tumourigenic dietary concentration of sulfoxaflor (500 ppm). At doses above 500 ppm, however, a dose-responsive increase in liver weight and hypertrophy

were demonstrated. Importantly, reversibility of hepatic hypertrophy (both liver weight and microscopic) was demonstrated in rats by a 90-day treatment with 1500 ppm sulfoxaflor, followed by a 28-recovery on control diet. The hepatic hypertrophic effects seen in both mice and rats following sulfoxaflor treatment are considered associative, supporting effects of the initial key event of CAR receptor activation for the MoA of CAR-mediated rodent liver tumourigenesis.

Key event #2 is an increase in hepatocellular proliferation and, consistent with the known MoA for CAR-mediated rodent liver tumourigenesis, the effects were seen in mice (both CD-1 and C57BL6 strains) and rats from a number of repeat-dose dietary studies (ranging from 3 to 90 days). Immunohistochemical staining for BrdU- or Ki-67-labeled nuclei as a measure of hepatocellular proliferation was based on interpretation of the nuclear staining intensity and an approximation of the location within the three hepatolobular zones (summarised herein; detailed in each individual report). For this MoA evaluation, panlobular (summed) counts were used for comparative purposes.

In the CD-1 male mouse, clear increases in hepatocellular proliferation were noted at the tumourigenic concentration of 750 ppm sulfoxaflor after seven days of administration, along with a similar response at a slightly lower concentration (500 ppm). Consistent with the known proliferative profile of PB (reviewed in *Cohen, 2010*), prolonged administration (28 to 90 days) of tumourigenic concentrations of sulfoxaflor resulted in a return to "normal" levels of hepatocellular proliferation. In the case of PB, the livers of treated animals were enlarged; however, stereologic studies indicate that hepatocellular proliferation is still enhanced compared to control animals due to the increase in the total number of hepatocytes per animal (*Lake, 2009*).

In the rat, 7 days of sulfoxaflor treatment induced a dose-responsive increase in hepatocellular proliferation, as measured by Ki-67 staining. While administration of 100ppm did not result in a significant increase in proliferation, a tumourigenic concentration of 750 or 1500ppm clearly increased the proliferative response of hepatocytes.

Evaluation of hepatocellular proliferation data from mice and rats over a range of study durations (3 to 90 days) and dietary concentrations of sulfoxaflor (100 to 2000 ppm) are informative as the second key event for rodent liver tumour induction associated with CAR activation. In addition, specificity of CAR-mediated, sulfoxaflor-induced hepatocellular proliferation in rodent liver was shown by experiments using CARKO/PXRKO and hCAR/hPXR mice. Specifically, in wild type C57BL6 mice, treatment of sulfoxaflor at 750ppm in the diet for 7 days induced a clear, statistically significant induction in the number of proliferating hepatocytes. This result was consistent with previous studies in rats and in the CD-1 strain of mice used in the combined chronic/carcinogenicity study, subchronic studies, and other MoA studies. Importantly, neither the CARKO/PXRKO or hCAR/hPXR mice had increased hepatocellular proliferation, underscoring the difference of rodent and human responses to CAR activation, and the qualitative differences in nuclear receptor-mediated hepatic responses.

Strength, consistency, and specificity of association of effects with key events.

Induction of cytochrome P450 enzyme activity is a well-known MoA for rodent hepatocarcinogens, and PB is a standard example of a CAR-mediated cytochrome inducer (*Whysner et al., 1996; Holsapple et al., 2006; Cohen, 2010*). The early key events for this MoA are: 1) CAR activation, with associated *CYP* isozyme induction, and 2) an increase in

hepatocellular proliferation that results in subsequent adenomas and carcinomas. In addition to these key events in the pathogenesis of hepatocellular tumours in rodents, reversibility of hepatic effects upon discontinuance of treatment is considered necessary data to support this MoA (*Cohen, 2010*).

When taken together the MoA and repeat-dose toxicity studies for both mice and rats described herein clearly demonstrate a sulfoxaflor-induced, robust, dose-related increase in the *Cyp2b*/CAR-associated transcript and associated increase in specific Cyp2b protein (Cyp2b10 in mice and Cyp2b1 in rats) and enzymatic activity (PROD/BROD). These results are consistent with the direct activation of the CAR nuclear receptor. In addition, analysis of hepatocellular proliferation indicates a clear, thresholded, dose-related induction of S-phase DNA synthesis. Both of these key events were demonstrated to be directly tied to the activity of the CAR nuclear receptor by the use of genetically modified mouse models (i.e., CAR/PXR-null, knockout, CARKO/PXRKO), where no CAR activity (gene or protein expression of Cyp2b10) or increase in hepatocellular proliferation was noted at a carcinogenic dose level of 750 ppm. Furthermore, the gross and microscopic hypertrophic effects of sulfoxaflor on the liver were reversible upon removal of the test material. Lastly, the *Cyp2b*/CAR-associated gene expression and protein data from these MoA experiments in both mice and rats define a very specific sulfoxaflor MoA while, simultaneously, rule out other nuclear receptor-mediated MoAs for rodent hepatic carcinogens such as PPAR-α or AhR agonism.

As previously summarised in the analysis of the MoA in this report, sulfoxaflor repeat dose dietary studies in mice and rats over a range of study durations and dose levels demonstrate a consistent dose and time association to the key events based on liver weights, microscopic hepatocellular hypertrophy, and hepatocellular proliferation data. Ultimately, taking into consideration both the mouse and rat chronic / cancer studies, as well as the shorter term studies, it is clear that nontumourigenic doses of sulfoxaflor were not associated with significant CAR activation, hypertrophy, hyperplasia, or any hepatocellular tumours while higher dose levels (>100ppm in the mouse and rat) resulted in clear instances of CAR activation, hypertrophy, hyperplasia, and hepatocellular tumours.

Overall the studies conducted with sulfoxaflor provide a strong and consistent association with sulfoxaflor-induced key events and a specific MoA that result in rodent hepatocellular tumours.

Biological plausibility and coherence. Dietary administration of sulfoxaflor to mice and rats results in the early key events (CAR activation and hepatocellular proliferation) that result in hepatocellular tumours after prolonged exposure to high dose levels of sulfoxaflor (750 ppm in mice and 500 ppm in rats). The early key events associated with hepatocellular hypertrophy are reversible upon cessation of treatment with sulfoxaflor. The MoA demonstrated for sulfoxaflor is consistent with the well-known MoA for PB in rodents (*Whysner et al., 1996; Holsapple, et al., 2006; Cohen, 2010*) and the MoA is consistent with current understanding of cancer biology and nuclear receptor-mediated carcinogenesis. The data for sulfoxaflor are entirely consistent with this non-genotoxic MoA in rodent liver.

In addition, the specificity for the MoA was demonstrated for sulfoxaflor using genetically engineered mouse models. As previously described, the CARKO/PXRKO mice were refractory to the CAR-mediated hepatic effects demonstrated for sulfoxaflor in wild type mice. Moreover, and most importantly, humanised CAR/PXR (hCAR/hPXR) mice demonstrated a similar, although quantitatively less, response for most endpoints directly

associated with CAR activation, but no increase in hepatocellular proliferation was noted. These data are consistent with the known MoA for PB and other CAR activators, and is considered to be supportive of why humans are refractory to the hepatotumourigenic effects of PB (*Holsapple et al., 2006; Cohen, 2010*).

Assessment of postulated sulfoxaflor mouse and rat liver tumour MoA.

The data for sulfoxaflor support a direct, threshold-based, dose-responsive MoA for hepatocellular adenomas and carcinomas in mice and rats. The MoA demonstrated for sulfoxaflor is consistent with the well-known MoA for PB in rodents (*Whysner et al., 1996; Holsapple, et al., 2006; Cohen, 2010*) and the MoA is consistent with the current understanding of cancer biology and nuclear-receptor mediated carcinogenesis. The data for sulfoxaflor are consistent with this non-genotoxic MoA in the liver. *In vitro* and *in vivo* studies show sulfoxaflor does not have a genotoxic MoA (see below).

The data for sulfoxaflor are judged with a high degree of confidence to adequately explain the development of hepatocellular tumours in mice and rats following chronic dietary administration.

Other possible modes of action for sulfoxaflor-induced rodent liver tumours.

As listed in table 6.5.3.6-1, the modes of action for hepatocellular carcinogenesis are broadly categorised as 1) DNA reactivity or 2) increased cell proliferation (i.e., mitogenic, which can be subcategorised as either receptor or nonreceptor mediated) (*Cohen, 2010*).

(1) DNA reactivity, mutagenicity and genotoxicity.

DNA reactivity is the second broad category of a MoA for hepatocellular carcinogens. There is no evidence from a comprehensive battery of genotoxicity assays of any mutagenic, clastogenic, aneugenic or DNA reactive activity of sulfoxaflor. A mutagenic mode of action is not supported. A battery of *in vitro* genotoxicity studies, the bacterial reverse mutation test (Ames test), mammalian chromosome aberration test, and a mammalian cell gene mutation test, all conducted in the absence or presence of a metabolic activation system (rat liver S9), showed that sulfoxaflor does not cause gene mutations or chromosome aberrations. Additionally, an *in vivo* mouse micronucleus assay showed that sulfoxaflor does not induce micronuclei in somatic cells. An evaluation of the genetic toxicity data for sulfoxaflor unequivocally supports no DNA reactivity and hence is not a potential MoA for the induction of hepatocellular tumours in mice and rats.

(2.a) Receptor mediated, increased cell proliferation.

MoAs for hepatocellular carcinogens that cause receptor-mediated hepatocellular proliferation include CAR, PXR, PPAR-α, and AhR agonism, as well as estrogens and statins (*Cohen, 2010*). The MoA studies in mice and rats with sulfoxaflor clearly demonstrate a specific, dose-related increase in the *Cyp2b*/CAR-associated transcript with associated increases in Cyp2b protein and enzymatic activity (PROD/BROD). Furthermore, CARKO/PXRKO animals further supported specificity for the sulfoxaflor-induced activation of the CAR/PXR receptor. Taken together, these findings are consistent with activation of the CAR/PXR receptor. At the same time, the MoA studies in mice and rats ruled out PPAR-α- and AhR-mediated nuclear receptor-mediated modes of action. Additionally, no indication of peroxisome proliferation was observed.

Oestrogens have a specific receptor-mediated MoA that results in cell proliferation in tissues including the liver; however, the carcinogenic activity may be due to an interaction of DNA adduct formation with increased cell proliferation (dual MoA) (*Cohen, 2010*). Sulfoxaflor is not likely to have an oestrogenic MoA based on structural dissimilarity to oestrogens and, in addition, there is no evidence of oestrogenic activity from a definitive two-generation toxicity study in the rat (*Rasoulpour et al., 2010*).

Statins act through inhibition of a specific enzyme, HMG-CoA-reductase, which leads to marked reduction in cholesterol production in humans (*Cohen, 2010*), but not in rodents (*Endo et al., 1970*). Epidemiologic evidence in human patients demonstrates that statins are not associated with an increase in liver or other tumours (*Farwell et al., 2008*). Thus, a statin MoA in rodents appears to be irrelevant to human carcinogenesis based on understanding the mechanism and extensive epidemiologic evidence (*Cohen, 2010*). Statins increase hepatocellular proliferation and hepatocellular tumours in rodents (*MacDonald and Halleck, 2004*); however, statins do not decrease serum cholesterol in rodents. The mode of action for statins is presumably due to an actual increase in liver HMG-CoA-reductase. Serum clinical chemistry values in rodents indicated that treatment with sulfoxaflor increased cholesterol. Although various classes of statins act differently, a common response is an increase in *Cyp2b* and *Cyp4a* gene expression and protein levels in the rodent liver (*Kocarek and Reddy, 1996*), whereas sulfoxaflor did not exhibit any induction in *Cyp4a* transcript levels, in fact in many cases it was decreased (table 6.5.3.6-2).

(2.b) Non-receptor mediated, increased cell proliferation.

MoAs for hepatocellular carcinogens that cause non-receptor mediated increased cell proliferation include cytotoxicity, infection, iron (copper) overload, and increased apoptosis (e.g., fumonisin B1) (*Cohen, 2010*). Cytotoxicity is unlikely to be a relevant MoA for sulfoxaflor as relevant toxicity data from numerous repeat-dose toxicity studies indicated a lack of any significant treatment-related necrosis and necrosis-related endpoints (i.e., huge increases in for example: alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase, etc.). In the studies that demonstrate notable individual cell necrosis and small elevations in ALT/AST, the dose levels where the effect occurred were at similar or higher levels than those that were associated with liver tumourigenesis. Furthermore, elevations in ALT/AST were associated with the 28- and 90-day studies in mice, whereas ALT/AST elevations were not noted in the rat repeat-dose studies. Taken together, the weight of evidence does not support a consistent association of cytotoxicity/necrosis in sulfoxaflor treated animals though there is a high dose effect where there is an increase in the incidence of very slight individual cell necrosis (apoptosis?). It is highly uncertain if this increase in individual cell necrosis is significant with respect to the eventual development of hepatocellular tumours.

An infectious MoA is not relevant for sulfoxaflor. Sulfoxaflor does not appear to result in hepatic accumulation of iron or copper based on histopathological findings in the liver for studies of multiple durations in either mice or rats, hence is not a potential contributing MoA for the rodent liver tumours. Sulfoxaflor did not appear to increase apoptosis in any of the previously described toxicity studies, although no direct measure was undertaken beyond standard histopathological analysis. In addition, as was previously described the CAR nuclear receptor-mediated MoA typically decreases apoptosis in initiated, proliferative foci of altered cells. Increased apoptosis for sulfoxaflor does not appear to be likely.

Sulfoxaflor rodent liver tumour human relvance framework:

Question 1. Is the weight of evidence sufficient to establish the MoA in animals?

The answer is yes. The MoA for sulfoxaflor-induced mouse and rat liver tumours is compatible with that described for PB-induced rodent liver tumours (*Whysner et al., 1996; Holsapple, et al., 2006; Lake, 2009; Cohen, 2010*). The relevant molecular and pathological endpoints for sulfoxaflor-induced liver effects in mice (*Geter et al., 2010; Ross, 2010*) and rats (*Geter and Kan, 2008; Geter and Card, 2010*) are supported by sulfoxaflor repeat-dose mouse and rat studies (*Yano et al., 2009a & b; Stebbins et al., 2010; Thomas et al., 2010a & b*), and there is good correlation for the dose response between the MoA data and the hepatocellular tumours. Key event #1 for the sulfoxaflor liver tumour MoA is defined as activation of the CAR/PXR nuclear receptor. This was measured by induction of the *Cyp2b10* transcript, protein, and associated liver enzyme activity (PROD/BROD). Supportive, associative data to key event #1 included increased liver weight and microscopic hepatocellular hypertrophy. Key event #2 is an increase in hepatocellular proliferation at the tumourigenic dose levels. Additionally, these key events demonstrated dependence on rodent CAR/PXR involvement, as CARKO/PXRKO or hCAR/hPXR animals did not respond similarly to wildtype mice to sulfoxaflor. When all the mechanistic and standard studies for sulfoxaflor are analysed, the key events show clear, thresholded, dose-responsive alterations and are consistent with a CAR-mediated MoA. In addition, other possible MoAs were examined and evaluated to be unlikely based on analysis of the relevant data for sulfoxaflor.

Question 2. Can human relevance of the MoA be reasonably excluded based on fundamental qualitative differences in key events between experimental animals and humans?

The answer is yes. Activation of the CAR nuclear receptor and subsequent induction of cytochrome P450 (CYP) enzyme activity in the liver following exposure to PB is a well known MoA for rodent hepatocarcinogenesis (*Whysner et al., 1996; Cohen, 2010*). The key events in CAR-mediated hepatocellular carcinogenesis include activation of CAR, induction of CYP isozymes, leading to increased hepatocellular proliferation with subsequent induction of proliferative lesions in the liver including foci, adenomas, and carcinomas. Activation of rodent CAR produces a cascade of alterations in gene transcription that leads to increased hepatocellular proliferation, a critical event in the development of liver tumours (*Whysner et al., 1996; Cohen, 2010*). On the other hand, PB in humans results in activation of CAR and PXR leading to the induction of CYP enzymes; however, different enzymes are induced in humans compared to rodents (*Lambert et al., 2009*) and, more importantly, there is no evidence of increased hepatocellular proliferation in humans or primary human hepatocytes *in vitro* (*Lake, 2009*). This finding was reinforced in the course of these studies with sulfoxaflor, where humanised CAR/PXR knock-in mice were refractory to the hepatocellular proliferative effect of sulfoxaflor, whereas wildtype mice demonstrated increased proliferation. Extensive epidemiologic studies in humans exposed to levels of PB comparable to those in rodent bioassays did not find an increased risk of cancer (*Whysner, et al., 1996; Lamminpaa et al., 2002*). Based on a MoA assessment, PB is not a hepatocarcinogen in humans. Furthermore, a hepatocarcinogenic response in rodents for compounds which have data to support a PB-like MoA is not relevant to humans (*Holsapple et al., 2006*). On this basis, the rodent liver tumours associated with administration of high dose levels of sulfoxaflor would not pose a cancer hazard to humans. A concordance analysis of the key events for the PB-like MoA (CAR activation) is presented in table 6.5.3.6-13.

Table 4.10.3.1.Study 6.13 (DAR Table 6.5.3.6-13). Concordance of Key Events for a

PB-like MoA in Rodents and Humans		
Key Event	**Evidence in Rodents**	**Evidence in Humans**
activation of CAR	yes	yes
CYP enzyme induction and liver hypertrophy	yes; unclear if critical step or indicator of activity secondary to CAR activation.	yes; different enzymes induced compared to rodents.
Hepatocellular proliferation	yes	No evidence of increased cell proliferation in the human liver (limited *in vitro* and *in vivo* data) or in "humanised" mouse liver experiments.
Selective clonal expansion (foci)	yes	No; none reported
Occurrence of hepatocellular tumours	yes	No; lack of any evidence based on human epidemiological data.

Question 3. Can human relevance of the MoA be reasonably excluded based on quantitative differences in either kinetic or dynamic factors between experimental animals and humans?

As human relevance of the experimental animal MoA can be reasonably excluded on the basis of qualitative differences in key events (Question 2); a quantitative assessment of kinetic or dynamic factors is not necessary.

Conclusions: prcd

Statement of confidence in the evaluation:

This Human Relevance Framework evaluation for sulfoxaflor-induced hepatocellular tumours in mice and rats follows the guideline established for this process (*Sonich-Mullin et al., 2001; Cohen et al., 2003; Meek et al., 2003; USEPA, 2005; Holsapple et al., 2006; Boobis et al., 2007*). The extensive toxicological database for sulfoxaflor, including several focused MoA studies in both mice and rats, as well as a study in genetically-engineered (knockout and humanised) mice are high quality studies that provide the necessary data to determine the MoA for sulfoxaflor-induced rodent liver tumours.

Key event #1 for the sulfoxaflor-induced liver tumour MoA is defined as activation of the CAR nuclear receptor, which is measured by the induction of *Cyp2b*/CAR-associated transcript (*Cyp2b10* in mice and *Cyp2b1* in rats), protein, and liver enzymatic activity. The *Cyp2b*/CAR-associated transcript and protein data define a very specific MoA while, at the same time, the data rule out several other potential nuclear receptor-mediated MoAs for rodent hepatic carcinogens such as PPAR-α and AhR agonism. PXR nuclear receptor-mediated *Cyp3a* cytochrome induction (*Cyp3a11* in mice and *Cyp3a3* in rats) was slightly induced after sulfoxaflor administration, analogous to the response after treatment with PB and consistent with the well documented co-activation of the receptors. Furthermore, these results were shown to be dependent on the rodent CAR and PXR nuclear receptors as knockout and humanised mice were not similarly responsive to sulfoxaflor treatment. Supportive, associative key events to #1 include increased liver weight and microscopic hepatocellular hypertrophy.

Key event #2 is an increase in hepatocellular proliferation and was indentified in both mice and rats. Importantly, neither the CARKO/PXRKO or hCAR/hPXR mice had increased hepatocellular proliferation, underscoring the difference of rodent and human responses to CAR activation, and the qualitative differences in nuclear receptor-mediated hepatic responses. The key events for sulfoxaflor show clear, threshold, dose-responsive alterations and provide informative, temporal-specific characterisation of sulfoxaflor-induced liver effects. These key events are consistent with a CAR-mediated (PB-like) MoA. The concordance analysis points out clear differences for a PB-like MoA in rodents as compared to humans. A hepatocarcinogenic response in rodents for compounds that have data to support a PB-like MoA, such as sulfoxaflor, is not relevant to humans (*Holsapple et al., 2006*).

Other possible MoAs for hepatocellular carcinogenesis as described by *Cohen* (2010) have been evaluated with respect to sulfoxaflor. Other MoAs due to increased cell proliferation (including receptor-mediated and non-receptor-mediated) or DNA reactivity have been dismissed for sulfoxaflor hepatocellular tumours because they lack plausibility and coherence or, in the case of cytotoxicity, because of the lack of coherence when the dose response for cytotoxicity is compared to the hepatocellular tumour dose response.

Identification of data gaps. Male mice and rats were more sensitive to the hepatic effects of sulfoxaflor and, hence, most of the mechanistic evaluations for MoA were performed in male mice and rats, including the studies with genetically engineered mouse models. Accordingly, the MoA/HRF evaluation described herein focused on the evaluation of the MoA in male mice and rats, although hepatocellular tumours at a lower incidence than that in their male counterparts were identified in female mice treated with a higher dietary concentration of 1250ppm for 18 months. Histopathological examination of the liver of those animals at dose levels with hepatocellular tumours (and of liver tissue in the shorter duration studies) revealed a phenotype entirely consistent with that identified in males of increased cytochrome P450 induction and eosinophilia. While inclusion of females in the MoA studies and MoA evaluation may have been informative, the MoA data provide compelling evidence that the sulfoxaflor liver tumour MoA is not sex specific but is sex selective in that males are more sensitive even at lower doses. Restricting the MoA investigations to the more sensitive sex significantly reduced the number of animals used for the studies.

Reversibility of sulfoxaflor-induced hepatic effects was investigated in a standard, repeat dose 90-day rat toxicity study. Animals administered the top dietary concentration of 1500ppm (i.e., 3-fold greater than the hepatocellular carcinogenic dose level in the 2-year rat study) for 90 days had a relative liver weight increase of 41% with clear microscopic hepatocellular hypertrophy identified. A subset of these animals were then subsequently switched to a control diet for an additional 28 days and the data indicated those animals did not have significantly increased relative liver weights or microscopic hepatocellular hypertrophy compared to control. A complete evaluation of the molecular reversibility for sulfoxaflor-induced hepatic effects across all MoA studies was not undertaken in an effort to restrict animal usage, as the most definitive experiment for specificity of sulfoxaflor-induced liver effects was demonstrated with the use of CARKO/PXRKO (knockout) and hCAR/hPXR (humanised) mice. The data from those animals demonstrated the molecular basis for the hepatocellular effects of sulfoxaflor.

Implications for risk assessment: There is convincing evidence that the MoA for sulfoxaflor-induced hepatocarcingenic effects in the mouse and rat liver do not occur below a defined dose level. Specifically, the MoA key events and hepatocellular tumours only occur at dietary

concentrations greater than 100 ppm in the mouse and rat, and tumours were noted at 500 and 750 ppm, respectively. Furthermore, a hepatocarcinogenic response in rodents for compounds that have data to support a PB-like MoA, such as sulfoxaflor, is not relevant to humans (*Holsapple et al., 2006*). These data were strengthened by the lack of hepatocellular proliferation in the CARKO/PXRKO and hCAR/hPXR mice. On this basis, the mouse and rat liver tumours associated with administration of higher dose levels of sulfoxaflor would not pose a cancer hazard to humans. Based on this hazard assessment for the sulfoxaflor-induced mouse and rat liver tumours, a margin of exposure risk assessment based on the reference dose (RfD) would be protective of human health.

Reliability of the study:

It was considered that the proposed mode of action (MOA) for the generation of liver tumours is plausible considering the data submitted. A MOA based on constitutive androstane receptor (CAR) activation was supported by the observation of increased Cyp2b enzyme expression and activation, increased liver weight, increased hepatocellular hypertrophy, and hepatocellular proliferation in both mice and rats. However, the use of the combined CAR/PXR knockout and hCAR/hPXR knockin mouse models does not delineate between CAR and PXR activities even though traditionally *Cyp2b* activity is primarily associated with activated CAR-mediated induction and *Cyp3a* activity is primarily associated with activated PXR-mediated induction. Significant overlap in the respective nuclear receptors ability to bind to DNA motifs and enhancer elements located in the regulatory regions and promoter sequences of either gene occurs and this has not been investigated in any detail. Nor has there been any investigation into the use of known species specific CAR/PXR activators with the transgenic mouse models employed (e.g. TCPOBOP for mouse CAR, 2,4,6-triphenyldioxane-1,3 – TPD for rat CAR, CITCO for human CAR, rifampicin for human PXR, pregnenolone-16α-carbonitrile – PCN for rat and mouse PXR). This would have helped to further strengthen the arguement for species specific CAR activity. The above noted effects are considered precursor events to liver tumour formation following a phenobarbital-like MOA, and such a MOA is not considered relevant to tumour formation in humans. Further, the observation of increased cell proliferation in wild type mice and the lack of a similar observation in CAR/PXR knockout and humanised mice is indicative of the specificity of the mouse CAR/PXR receptors' role in inducing the necessary precursor event of cell proliferation. The observation of all precursor key events was assessed at the turmourigenic dose in mice. Cytochrome 2b enzyme induction and expression and cell proliferation was only assessed (and observed) at a dose level above the tumourigenic dose in rats (750 ppm vs. 500 ppm). Nonetheless, all precursor events have general temporal and dose concordance with the observation of liver tumours.

Limited liver cytotoxicity by way of increased incidences of individual hepatic cell necrosis, scored as very slight in nature and observed in a number of studies may be correctly described as treatment related effects (90-day dietary studies in the rat and mouse, single-cell hepatocyte necrosis was observed at≥750 ppm (47 .6 and 98 mg/kg bw/day); 2-generation reproduction study, very slight centrilobular single cell necrosis of the liver in parental male Sprague-Dawley rats at the high dose of 400 ppm (24.6 mg/kg bw/day); in a mode of action study investigating liver weight effects in CD-1 mice, single cell necrosis was observed in males at 500 ppm (89 mg/kg bw/day) and above). However, these effects were generally seen at the tumourigenic dose in both rats and mice≥ 500ppm). Though initially this observation may not be consistent with a phenobarbital-like MoA, there was no evidence for extensive liver cytotoxicity from other histological indices or clinical chemistry. There were no significant elevations in plasma hepatic transaminases to warrant concern for cytotoxicity as a major

modus operandi for liver tumour development. It was not considered that the present available evidence is sufficient to suggest that sulfoxaflor may operate via more than one primary mode of action to induce liver tumours in rodents. Both activation of the CAR as well as some limited induction of cytotoxicity in the liver are occurring concordant with liver tumours but the weight of evidence from all the studies would suggest the primary activity is CAR/PXR activation followed by liver enzyme induction, hepatocyte proliferation with subsequent induction of proliferative lesions in the rodent liver including foci, adenomas, and carcinomas. Initial short term events such as CAR-dependent enzyme induction, liver weight increases and hepatocyte proliferation differ depending on the genetic constitution of the CAR/PXR nuclear receptors in mouse transgenic models and support the hypothesis that species-specific CAR activation is the probable cause of the liver tumours observed in the rodent studies at high concentrations of sulfoxaflor. In addition, there is no concern for mutagenicity. Neither sulfoxaflor nor its metabolites caused gene mutations or chromosome aberrations in *in vivo* or *in vitro* studies. In conclusion, the evidence supports a non-genotoxic, threshold based, mitogenic response similar to a phenobarbital (PB) like MoA for these rodent liver tumours.

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Study 7: MoA Study: Rat/F344 and Crl:CD(SD); (testosterone elimination and dopamine agonism and / or enhancement MoA study. DAR Section B.6.5.4.1.

Increased size of Leydig cell adenomas was seen in the testes of male F344 rats given 4.24 or 21.3mg/kg/day (100 or 500ppm) in the rat long term carcinogenicity study (B.6.5.1.1). The overall incidence of animals with interstitial cell adenomas was not increased at any dose level but the incidence of animals with tumours in both testes was significantly increased ($P<0.05$) at the high dose level. Several mechanisms by which this might happen were described but three in particular (reduced testosterone biosynthesis, increased testosterone biliary elimination and dopamine agonism/enhancement which interfers with Leydig cell control mechanisms at a variety of points along the hypothalamic/pituitary/testicular axis.) were tested in an in vivo Leydig cell MoA study (section B.6.5.4.1; Rasoulpour, 2010a). The data generated did not support increased testosterone biliary elimination nor reduced testosterone biosynthesis but provided some evidence supporting dopaminergic activity in the form of decreased circulating Prl levels, with increased LH and T levels, along with decreased testis LHR gene expression. It was thought highly plausible that this MoA could operate through sulfoxaflor-mediated enhancement of dopamine release, potentially though agonism of α4β2 or α4α6β2 mammalian central (neuronal) nicotinic acetylcholine receptors (nAChRs), which are known to play a key regulatory role in dopamine release from dopaminergic neurons in the brain.

F344 and Crl:CD(SD) rats were treated with 0, 25, 100 or 500ppm sulfoxaflor for up to 8 weeks. Gene expression analysis of testes mRNA from this study was conducted on a suite of

steroidogenic enzymes to evaluate reduced testosterone biosynthesis. There was no dose-dependent effect of treatment on any measured gene in the steroidogenic pathway including StAR (steroidogenic acute regulatory protein), Cyp11a1 (P450side chain cleavage), Cyp17a1 (17alpha-hydroxylase), HSD3b (3-beta hydroxysteroid dehydrogenase), or SDR5a1 (5-alpha reductase). If reduced testosterone biosynthesis were the operant MoA, one or more of these genes would be affected. Furthermore, the hormone panel data would have shown a sustained decrease in circulating levels of testosterone, which was not observed in the LCT MoA study (*Rasoulpour et al., 2010a*).

Support for increased testosterone biliary elimination would be visualised by a dose-dependent increase in the amount of Testosterone-derived radioactivity eliminated in the bile. However, there were no statistically significant (alpha = 0.05) or treatment-related differences in the mean 14C-testosterone-derived radioactivity excreted in the bile across all dose groups, per time intervals, for F344/DuCrl rats. In addition the blood hormone panel data would have shown a decrease in circulating levels of testosterone, which was not observed in either strain at any time point.

Subsequently, in vitro data were generated to refute other potential MoAs. The data refuted androgen receptor antagonism (sulfoxaflor was negative for AR agonism and antagonism), oestrogen receptor agonism/antagonism (sulfoxaflor was negative for ER binding and ER agonism and antagonism) and aromatase inhibition (sulfoxaflor was negative for CYP19 inhibition).

Report: Rasoulpour, R. J., Zablotny, C. L., Clark, A. J., Hansen, S. C., Zhang, F. (2010). XDE 208: Leydig Cell Mode-of-Action Study in Crl:CD(SD) and F344/DuCrl Rats. Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan, 48674. Unpublished.

Report No.: DECO HET DR-0404-3134-115. Study ID: 101105.

Dates: 2010

Guidelines: Non-guideline.

GLP: Yes. All experiments were done according to GLP standards and are fully reliable even though the study is not GLP compliant.

Deviations: This is an acceptable though non-guideline study, it is considered supplementary to the long-term chronic / carcinogenicity studies.

Deficiencies: Yes, a group of positive control animals treated with a known and well documented dopamine agonist (DA) such as mesulergine would have provided the appropriate positive data to relate results from sulfoxaflor treated animals and therefore give a better understanding into the actions of sulfoxaflor. This would help to determine if sulfoxaflor operated in a similar manner to a DA.

Executive Summary: In a recently conducted two-year rat carcinogenicity study, male Fischer 344 rats given 100 or 500 ppm sulfoxaflor had a treatment-related increase in testis weight due to increased Leydig cell tumour (LCT) size. Histopathological examination confirmed that there was no increase in the overall incidence of LCT across the groups with 88, 92, 90, and 92% of rats affected at 0, 25, 100, and 500 ppm, respectively. However, there was a significant increase in bilateral LCT incidence at 500 ppm. The objective of this study

was to identify the mode-of-action (MoA) responsible for these Leydig cell effects, also to determine if the MoA operated in Crl:CD(SD) rats, the strain used in the two-generation reproductive toxicity study where an apparent slight delay in preputial separation was seen at the high dose level of 400ppm.

General modes of action for rat Leydig cell tumours: It is generally accepted in the literature that there are nine known modes-of-action for Leydig cell tumour induction in rats, which fall into three 'bins' of human relevance (i.e., relevant, low relevance, no relevance). These are:

Relevant to humans:	(1) mutagenicity
Low relevance to humans:	(2) androgen receptor antagonism
	(3) oestrogen receptor agonism/antagonism
	(4) 5-alpha-reductase inhibition
	(5) aromatase inhibition
	(6) **reduced testosterone biosynthesis**
	(7) **increased testosterone biliary elimination**
No relevance to humans:	(8) GnRH (LHRH) agonism
	(9) **Dopamine agonism/enhancement**

Relevant modes of action for sulfoxaflor-induced LCTs: The only relevant modes of action for sulfoxaflor considered to operate are those points emboldened above (MoA #6, #7, and #9). The suite of toxicity studies on sulfoxaflor, from a battery of genetic toxicity assays to developmental and reproductive toxicity to chronic/carcinogenicity studies, provides evidence that either refutes or cast significant doubt on the plausibility of a number of the other MoAs. For example, MoA #1 (mutagenicity) is not plausible as sulfoxaflor was negative in all *in vitro* and *in vivo* genetic toxicity assays. In addition, MoA #2 – 5 and #8 are also not plausible as there were no effects on end points that would have been affected with these MoA, such as male anogenital distance, accessory sex gland weights, mating or fertility indices, vaginal patency, or pituitary effects.

Reduced testosterone biosynthesis as a primary effect (#6) was deemed to have low plausibility as there was an increase in serum cholesterol levels with sulfoxaflor administration and a slight delay in preputial separation; however, there was no effect on female reproductive parameters, which would have been expected with this MoA as androgens are the precursors to oestrogens. In support of MoA #9, a prototypical dopamine agonist/enhancer, such as mesulergine, would cause a delay in preputial separation as well as decreased levels of circulating Prl (*Prentice et al., 1992*). Despite the relatively low plausibility, an assessment of steroidogenic gene expression was performed in this study to evaluate the reduced testosterone biosynthesis MoA.

The two most plausible MoAs, which both had a detailed analysis in this LCT MoA study, were increased biliary elimination of testosterone (#7) and dopamine agonism/enhancement (#9). MoA #7 was deemed plausible due to known nuclear receptor-mediated liver effects of sulfoxaflor, which could result in increased biliary elimination of testosterone and a compensatory increase in luteinizing hormone (LH) release from the pituitary gland. Trophic

stimulation of the rat Leydig cells by persistently higher levels of circulating LH would, over time, lead to formation of Leydig cell tumours (*Cook et al., 1999*). MoA #9 was deemed plausible because sulfoxaflor is an agonist to the foetal rat muscle nicotinic acetylcholine receptor (nAChR) (*Millar, 2010*), the molecular target for insecticidal activity is the nAChR, and mammalian central nAChRs are known to play a key regulatory role in dopamine release in the brain (*Maskos, 2010*). The dopamine agonism/enhancement MoA occurs via antagonist action of dopamine on prolactin (Prl) release in the pituitary gland (*Cook et al., 1999*). Lower circulating Prl results in decreased prolactin binding on rat Leydig cells, which results in down-regulation of the LH receptors (*Prentice and Miekle, 1995*). This, in turn, results in transient decrease in circulating testosterone, which feeds back to stimulate an increase in LH release from the pituitary. As with MoA #7, chronic LH stimulation can lead to Leydig cell hyperplasia and eventually tumour formation.

Groups of 15 Fischer 344 and 15 Crl:CD(SD) rats were given 0, 25, 100, or 500ppm sulfoxaflor in diet (120 total animals) for up to 8 weeks. After two weeks of treatment, three rats / group were selected for the biliary elimination of testosterone (#7) portion of the study. Briefly, bile duct cannulated rats were injected with ^{14}C-testosterone followed by bile and plasma collection over a two-hour period to determine if sulfoxaflor treatment altered the biliary elimination profile. In order to directly test if dopamine agonism / enhancement (#9) was the responsible MoA, a serum hormone panel of testosterone (T), luteinizing hormone (LH) and prolactin (Prl) were evaluated on all available animals after 2, 4, and 8 weeks of treatment. In addition to hormone measurements, gene expression analysis for LH receptor (LHR) and Prl receptor (PrlR) was performed on testes of 4- and 8-week treated Fischer rats. To directly test if reduced testosterone biosynthesis (#6) was the responsible MoA, gene expression of critical steroidogenic enzymes StAR (steroidogenic acute regulatory protein), Cyp11a1 (P450side chain cleavage), Cyp17a1 (17alpha-hydroxylase), HSD3b (3-β hydroxysteroid dehydrogenase), and SDR5a1 (5-α reductase) were evaluated in 4- and 8-week Fischer rat testes. If reduced testosterone biosynthesis was the operant MoA, one or more of these genes would be affected.

Results from the biliary elimination portion of this study revealed no treatment-related differences in the mean ^{14}C-testosterone derived radioactivity excreted in the bile, levels in circulating plasma, or in bile flow for Crl:CD(SD) and Fischer rats. This refutes (#7) as the operant MoA. Reduced testosterone biosynthesis (#6) had low plausibility due to the fact that female reproductive parameters were not affected in any study, including the two-generation reproductive toxicity study. There were no effects such as altered oestrous cyclicity, mating and fertility indices. There were no dose-dependent effects of treatment on any measured gene in the steroidogenic pathway including *StAR*, *Cyp11a1*, *Cyp17a1*, *HSD3b*, or *SDR5a1*. If reduced testosterone biosynthesis was the operant MoA, one or more of these genes would have been affected. The data presented in this study provide evidence supporting (#9) in the form of decreased circulating Prl levels, with increased LH and T levels, along with decreased testis LHR gene expression. The observation of hormone level alterations in this study support a hormonally-mediated, and thereby threshold, nonlinear mode-of-action. This MoA is hypothesised to operate through sulfoxaflor-mediated enhancement of dopamine release, potentially though agonism of α4β2 or α4α6β2 central nicotinic acetylcholine receptors (nAChRs), which are known to play a key regulatory role in dopamine release from dopaminergic neurons in the brain.

It is hypothesised that the LCT promotion seen in the rat chronic/carcinogenicity study was through weak, but chronic, enhancement of dopamine release, and subsequent inhibition of prolactin release from the pituitary gland, ultimately leading to a dopamine

agonism/enhancement LCT MoA in a uniquely susceptible animal model, the Fischer 344 rat. This MoA would be considered to have no relevance to humans, per se.

Materials and Methods

Materials:

1	**Test Material:**	Sulfoxaflor
	Synonyms:	XDE-208; (N-(Methyloxido(1-(6-(trifluoromethyl)-3-pyridinyl)ethyl)-λ^4-sulfanylidene)-cyanamide); [1-(6-Trifluoromethylpyridin-3-yl)ethyl](methyl)-oxido-λ^4-sulfanylidenecyanamide; Sulfoximine; X11422208; XR-208.
	Description:	White Solid
	Lot/Batch #:	Lot # E2162-34, TSN003725-0001.
	Purity:	95.6% (w/w); as two diastereomers in 50.4 / 49.5% ratio
	Contaminants:	
	CAS #:	946578-00-3
2	**Vehicle:**	LabDiet Certified Rodent diet #5002 (PMI Nutrition International, St. Louis, Missouri, US)
	Dose	Ingested via the oral (dietary) route at 0, 25, 100 or 500ppm. These equated to time-weighted average doses of: F344/DuCrl males; 0, 1.41, 5.58 and 27.8 mg/kg bw/day, Crl:CD(SD) males; 0, 1.37, 5.59 and 27.7 mg/kg bw/day.
3	**Test Animals:**	
	Species:	Rat
	Strain:	F344/DuCrl males and Crl:CD(SD) males.
	Age/weight at study initiation:	12 weeks / F344/DuCrl males, 239.1 – 240.1g; Crl:CD(SD) males, 368.2 – 370.5g
	Source:	Charles River Laboratories (Kingston, New York) - F344/DuCrl rats; Charles River Laboratories (Portage, Michigan) - Crl:CD(SD) rats
	Housing:	After assignment to study, animals were housed singly in stainless steel cages. Cages had wire mesh floors and were suspended above catch pans. Non-woven gauze was placed in the cages to provide a cushion from the flooring for rodent feet and also provided environmental enrichment. Cages contained a feed crock and a pressure activated lixit valve-type watering system.
	Feed and Water:	Feed and municipal water were provided *ad libitum*. Analyses of the feed were performed by PMI Nutrition

	International to confirm the diet provides adequate nutrition and to quantify the levels of selected contaminants. Drinking water obtained from the municipal water source.
Environmental conditions:	**Temperature:** 22 ± 1°C **Humidity:** 40-70% **Air Changes:** 12-15 times/hour **Photoperiod:** 12-hour light/dark
Acclimation period:	The rats were acclimatised for a period of 1 week prior to the start of the study.

Study Design:

1. In life dates: The study was initiated on 19 July 2010. No further information provided.

2. Animal assignment and treatment groups: Before administration of test material began, animals were stratified by body weight and then randomly assigned to treatment groups. Animals placed on study were uniquely identified via subcutaneously implanted transponders (BioMedic Data Systems, Seaford, Delaware) that were correlated to unique alphanumeric identification numbers.

Groups of 15 Fischer 344 and 15 Crl:CD(SD) rats were given 0, 25, 100, or 500 ppm sulfoxaflor in the diet (120 total animals) for up to 8 weeks. In order to directly test #7 (enhanced biliary elimination of testosterone), 3 rats/group underwent bile cannulation after 2 weeks of treatment to measure levels of ^{14}C-testosterone-derived radioactivity within the bile and flow rate of the bile over a two hour period. In order to directly test #9 (dopamine agonism/enhancement), a serum hormone panel of testosterone (T), luteinizing hormone (LH), prolactin (Prl), and 17β-estradiol (E2), were evaluated on all available animals after 2, 4, and 8 weeks of treatment. In addition to hormone measurements, analysis of gene expression levels on LH and Prl receptors in all Fischer 344 rat testes (4- and 8-week) as well as immunohistochemistry of LH receptors (LHR) in the testes of all necropsied rats at 4 weeks was performed. Quantification of LHR immunostaining was performed only on Fischer 344 rat testes (4-week only). During the 4- and 8-week necropsies, liver samples collected for possible analysis were, deemed not necessary based on results from the biliary elimination portion of the study indicating the liver was not involved in the MoA. In addition, portions of testis were also frozen during these necropsies as contingencies for immunohistochemistry on frozen sections, which were not necessary as this was performed on formalin fixed tissue.

The key study parameters (table 6.5.4.1-1) and study schedule were:

2 weeks (14 days):	serum hormone panel {T, E2, Prl, LH} 7 – 12 rats per group
	bile cannulation {T_{bile}, T_{plasma}} 3 rats per group
4 weeks (30 days):	serum hormone panel {T, E2, Prl, LH} 7 – 12 rats per group
	interim necropsy on 4 rats /group

	testis collection {LH & Prl receptor gene expression, LH receptor immunohistochemistry}
8 weeks (60 days): group	serum hormone panel {T, E2, Prl, LH} remaining 3 – 8 rats per
	terminal necropsy on 8 rats /group
	testis collection {LH & Prl receptor gene expression, LH receptor immunohistochemistry}

Table 4.10.3.1.Study 7.1 (DAR Table 6.5.4.1-1): Summary of Key Study Parameters and Study Schedule

Study Events and Parameters	No. Animals	Timing
Cage-side examinations	All	At least once daily
Clinical observations	All	Weekly
Body weights –males	All	pre-exposure period; weekly; at termination
Feed consumption –males	All	Weekly
Hormone analysis	Up to 12/strain/group	2, 4 and 8 weeks
Bile cannulation	3/strain/group	2 weeks
Gross necropsy – adult males	4/strain/group	4 weeks
Gross necropsy – adult males	8/strain/group	8 weeks
Organ weights – adults	12/strain/group	At necropsy

3. Diet preparation and analysis: Diets were prepared by serially diluting a concentrated test material-feed mixture (premix) with ground feed. Diets were prepared as a fixed percent of test material in rodent feed. The test material concentration was not adjusted for purity. Premixes and diets were prepared periodically throughout the study based on stability data. Analyses of the low-dose male and high-dose male diet indicated that the test material was homogeneously distributed. Analyses of all test diets from the first mix of the main study revealed mean concentrations ranging from 87.7 to 100.5% of targeted concentrations indicating an acceptable concentration of sulfoxaflor was achieved.

4. Statistics: Body weights, feed consumption, serum hormone levels, and organ weights (absolute and relative) were first evaluated by Bartlett's test (alpha = 0.01) for equality of variances. Based upon the outcome of Bartlett's test, either a parametric or non-parametric analysis of variance (ANOVA) was performed. If the ANOVA was significant at alpha = 0.05, a Dunnett's test (alpha = 0.05) or the Wilcoxon Rank-Sum (alpha = 0.05) test with Bonferroni's correction was performed. Bile cannulation and immunohistochemistry data were run by a parametric analysis of variance (alpha = 0.05;). Feed consumption values were excluded from analysis if the feed was spilled or scratched. Statistical outliers (alpha = 0.02)

were identified by the sequential method of *Grubbs* (1969) and were routinely excluded from feed consumption only. Other outliers, if excluded, were excluded from analysis for documented, scientifically sound reasons.

Methods:

Observations: A cage-side examination was conducted at least once a day. This examination was typically performed with the animals in their cages and was designed to detect significant clinical abnormalities that were clearly visible upon a limited examination, and to monitor the general health of the animals. The animals were not hand-held for these observations unless deemed necessary. In addition, all animals were observed for morbidity, mortality, and the availability of feed and water at least twice daily. Clinical observations were conducted on all males pre-exposure and weekly throughout the study.

Body weight: All rats were weighed pre-exposure, and weekly thereafter and at termination.

Food consumption and compound intake: Feed consumption was determined for all animals by weighing feed containers at the start and end of a measurement cycle. The compound intake was calculated using test material concentrations in the feed, actual body weights (BW) and measured feed consumption data. Feed consumption was determined at least weekly.

Clinical Chemistry: Serum hormone parameters were measured in 7-12 rats/group after two weeks of treatment, 5-12 rats/group after four weeks of treatment and 3-8 rats/group after eight weeks of treatment. Non-fasted animals had 600µl blood collected from the jugular vein (~ 8:00 AM) without the use of anaesthesia. Serum samples were frozen on dry ice and kept at -80°C until LCMS analysis. A 100µl aliquot of serum was used to determine concentration of testosterone (T), and 17β-oestradiol (E2) at TERC by LCMS. A different 150µl sample was shipped to Ani Lytics Inc to determine prolactin (Prl) concentration conducted according to GLP procedures by radioimmunoassay (RIA). In addition, pooled samples to result in approximately 50µl of serum were sent to Ani Lytics Inc to determine the concentration of luteinizing hormone (LH) according to GLP procedures by RIA. Lastly, terminal samples of (4/group at 4 weeks and 8/group at 8 weeks) were sent to Ani Lytics Inc to determine the concentration of LH, Prl and T according to GLP procedures by RIA.

Jugular Vein and Bile Duct Cannulation: Three non-fasted animals per group per strain (control, 25ppm, 100ppm, and 500ppm) were anaesthetised using isoflurane and surgery was performed to cannulate the jugular vein and bile duct. After the establishment of a consistent bile flow, each rat received an intravenous (*i.v.*) administration of ^{14}C-testosterone in (0.8µCi/rat at 57mCi/mmol) via the jugular vein. After administration, the rats were placed on a warming pad to maintain body temperature. Blood samples (100µl) were taken from jugular vein cannula and/or via orbital sinuses at 5, 15, 45, 75, and 105 minutes post-injection. Plasma was prepared and a weighed aliquot analysed by liquid scintillation spectroscopy (LSS) to determine the amount of ^{14}C-radioactivity. Any remaining plasma samples were stored at -80°C for possible future use. Bile samples were collected over a 2-hour interval (30, 60, 90, and 120 minutes post-injection) in tared vials. The weight of the bile per time interval was determined. A weighed aliquot of bile per time interval were analysed for ^{14}C testosterone-derived radioactivity by LSS. The total amount of radioactivity in those intervals was determined. After bile collection, animals were euthanized via cervical dislocation. No organs were collected from these animals.

Targeted Gene Expression: A portion of the right testis and appropriate liver samples were

preserved in RNAlater from all exposure groups. Liver samples were not processed or analysed based on results from the biliary elimination portion of the study. Total RNA was extracted from testes using the Qiagen RNeasy kit following the manufacturer's protocol. RNA quantity and quality were assessed by a NanoDrop ND-1000 spectrophotometer and Agilent 2100 Bioanalyzer, respectively. Only samples with an OD 260/280 ratio greater than 1.8 and with clearly defined 28S and 18S bands were used for targeted gene expression studies. Total RNA was treated with DNase enzyme to avoid DNA contamination. Targeted gene expression studies were conducted using an Applied Biosystems 7500 real-time Polymerase Chain Reaction system using Applied Biosystems TaqMan Gene Expression Assays. Due to the nature of the TaqMan system, dissociation curves were not required to verify the specificity of the PCR reactions.

The following genes in the testis were selected to investigate sulfoxaflor effects:

1. *Lhcgr* - Luteinizing hormone/choriogonadotropin receptor,
2. *Prlr* - Prolactin receptor,
3. *StAR* - Steroidogenic acute regulatory protein,
4. *Cyp11a1* - Cytochrome P450, family 1, (mitochondrial P450scc)
5. *Srd5a1* - Steroid-5-α-reductase,
6. *Cyp17a1* - Cytochrome P450, family 17, (17α hydroxylase/C17, 20 lyase)
7. *Hsd3B* - 3β-hydroxysteroid dehyrogenase.

Immunohistochemistry: One testis from each rat selected for the 4-week necropsy time point was cross-sectioned, placed in a labelled tissue cassette, and immersed in 10% neutral buffered formalin. The tissue remained in fixative for approximately 72 hours and then processed on an automated tissue processor. Tissue blocks were embedded in paraffin and sectioned on a rotary microtome. Immunohistochemistry was used for the detection of the luteinizing hormone receptor (LHR) in the testis sections. Quantification of LHR immunostaining was performed on all 4-week Fischer 344 rat testes. As the largest hormone changes were observed at the 4-week time point, these slides were blinded and quantified for number of Leydig cells with intracellular staining of LH receptor out of 1000 Leydig cells counted in two cross-sections of tissue.

Sacrifice and pathology: Adult males (non-fasted) were submitted for necropsy after at least 4 and 8 weeks of exposure. The animals were weighed in the animal room on the morning of the scheduled necropsy. The animals were anaesthetised by the inhalation of CO_2/O_2 and blood was collected from the orbital sinus. Their tracheas was exposed and clamped, and the animals were euthanised by cervical dislocation. Weights of the liver and testes were recorded, and organ:body weight ratios calculated. Animal 7205 died during blood collection at the 2-week time point due to complications associated with the jugular vein blood collection procedure; therefore, a necropsy and gross pathological examination were not performed on this animal.

Results and Discussion

Observations

Clinical signs of toxicity:

Examinations performed on all animals prior to the study revealed that all animals were in good health for study purposes. Examinations performed on all animals weekly throughout the study revealed no treatment-related findings.

Mortality:

Animal 7205 died during blood collection at the 2-week time point due to complications associated with the jugular vein blood collection. There were no sulfoxaflor associated deaths.

Body weight and body weight gain

Administration of sulfoxaflor had no effect on the bodyweight (table 6.5.4.1-2) over the 8 week period of the study. There was no loss of body weight during the study and similar body weight gains were seen throughout the study.

Table 4.10.3.1.Study 7.2 (DAR Table 6.5.4.1-2): Selected intervals for mean body weights of both male strains.

Dose ppm	F344				SD			
	0	25	100	500	0	25	100	500
Initial wt.	240.1	239.1	239.9	239.9	370.0	369.5	368.2	370.5
day 15	263.3	265.2	266.2	262.9	435.6	434.4	428.2	427.4
day 29	274.6	280.2	280.4	281.3	476.3	465.1	457.2	459.8
day 57	303.4	315.2	316.2	315.1	555.7	516.7	527.1	518.7

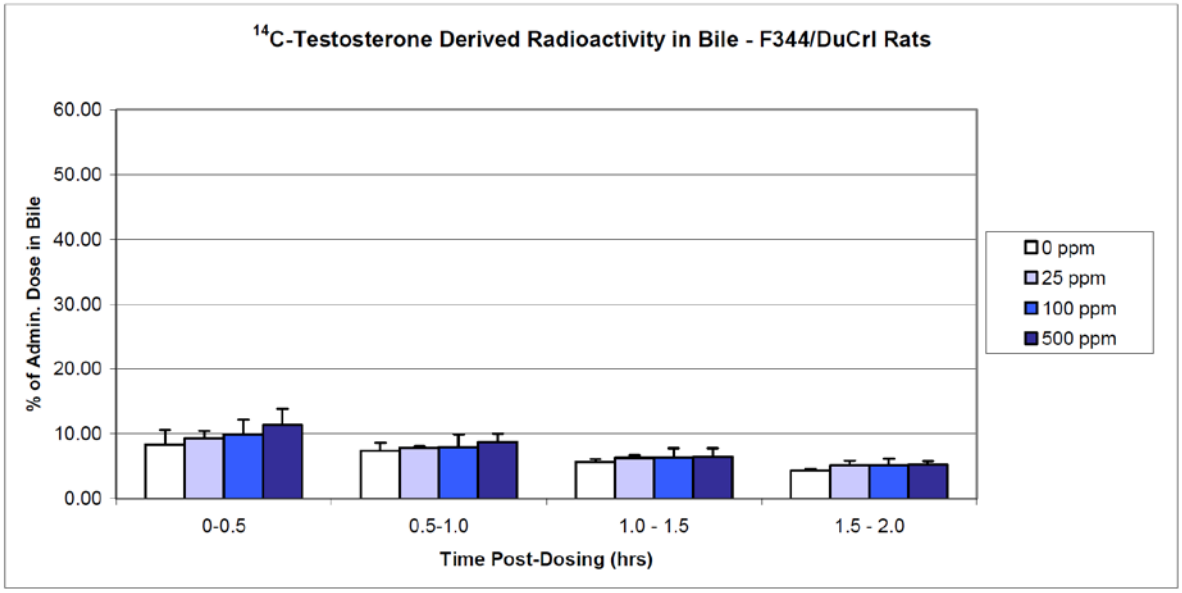
Food consumption and compound intake

There were no treatment-related effects on feed consumption in either animal strain during the study. Time weighted average doses for animals given 0, 25, 100, or 500ppm sulfoxaflor were 0, 1.41, 5.58, and 27.8 mg/kg/day in F344/DuCrl rats; 0, 1.37, 5.59, and 27.7 mg/kg/day in Crl:CD(SD) rats.

Clinical chemistry

1. Biliary elimination of testosterone:

In order to test #7 (enhanced biliary elimination of testosterone), Fischer 344 and Crl:CD(SD) rats underwent bile cannulation after two-weeks of treatment with sulfoxaflor. Animals were injected with radiolabeled testosterone (T) in order to most accurately track plasma and biliary levels of testosterone and any potential metabolites over two hours.


14C-Testosterone Derived Radioactivity in Bile - F344/DuCrl Rats
% of Admin. Dose in Bile
60.00
50.00
40.00
30.00
20.00
10.00
0.00
0-0.5
0.5-1.0
1.0 - 1.5
1.5 - 2.0
Time Post-Dosing (hrs)
0 ppm
25 ppm
100 ppm
500 ppm

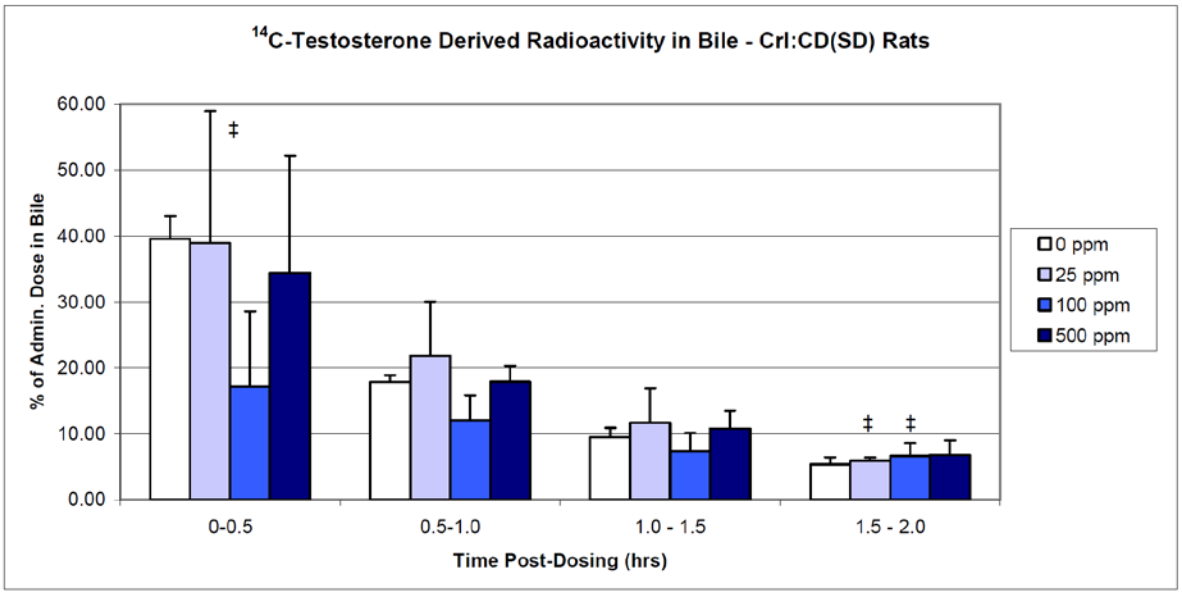


Figure 4.10.3.1.Study 7.1 (DAR Figure 6.5.4.1-1): ^{14}C-Testosterone derived radioactivity in bile from F344/DuCrl and Crl:CD(SD) rats.

Experimental support for #7 would show a dose-dependent increase in the amount of T-derived radioactivity eliminated in the bile. However, there were no statistically significant (alpha = 0.05) or treatment-related differences in the mean ^{14}C-testosterone-derived radioactivity excreted in the bile across all dose groups, per time intervals, for Crl:CD(SD) and F344/DuCrl rats (figure 6.5.4.1-1). Bile flow was very similar for the respective dose groups, time intervals and strains. Overall, Crl:CD(SD) rats excreted approximately 1.5 to 3 times the cumulative amount of bile ^{14}C-testosterone-derived radioactivity, than F344/DuCrl rats from the respective dose groups. The lower plasma radioactivity values for Crl:CD(SD) rats are consistent with having a higher biliary clearance than F344/DuCrl rats. Taken together, these data refute #7 (biliary elimination of testosterone) as the operant MoA.

2. Serum hormone levels:

A hormone panel was performed at 2-, 4-, and 8-weeks of treatment from both Fischer 344 and Crl:CD(SD) rat serum from in-life bleeds, as well as terminal samples from the 4- and 8-week necropsy time points (tables 6.5.4.1-3 and 6.5.4.1-4). Serum concentrations of 17β-oestradiol (E2) were analysed by LC/MS; however, all but two of these values were below the lower limit of quantitation, and are therefore not reported. Due to the inherent variability within hormone measurements, particularly pulsatile hormones such as LH, the data are interpreted along with dose-response relationships and concordance with other end points.

There was no effect of treatment on Fischer rat hormone levels at the 2- or 8-week time points; however, at 4-weeks there was ~2-fold dose-dependent increase in luteinizing hormone (LH) levels concomitant with ~1.7-fold dose-dependent decrease in prolactin (Prl) levels. This increase in serum LH was accompanied by ~3-fold dose-dependent increase in Testosterone (T). This hormone profile was somewhat recapitulated in Crl:CD(SD) rats, albeit with a different timing, where an increase in LH occurred with a concomitant increase in T at 2-weeks, and a decrease in Prl occurred at 4-weeks.

Table 4.10.3.1.Study 7.3 (DAR Table 6.5.4.1-3): Mean serum hormone levels in both male strains from in-life bleeds.								
Dose	**F344**				**SD**			
ppm	**0**	**25**	**100**	**500**	**0**	**25**	**100**	**500**
2 weeks								
Prl	9.48	12.78	8.16	9.42	14.61	15.71	11.22	15.13
T	0.76	0.83	0.54	0.90	2.01	**4.49**	3.67	**3.70**
LH	0.54	0.81	0.27	0.42	0.29	0.49	0.36	**0.78**
4 weeks								
Prl	17.89	15.48	16.18	10.39	11.56	11.23	10.26	8.97
T	0.67	1.00	1.19	0.93	2.42	2.61	4.67	2.50
LH	0.47	0.54	0.66	**0.88**	1.10	0.34	0.36	0.35
8 weeks								
Prl	19.34	17.94	19.45	17.53	14.50	16.46	24.33	19.10
T	0.58	0.67	0.77	0.70	1.75	2.74	2.43	1.94
LH	0.89	1.04	0.69	1.08	0.25	0.47	**0.66**	0.50

Prl = prolactin, ng/ml; T = testosterone, ng/g; LH = luteinizing hormone, ng/ml. 2 weeks treatment, n = 7 – 12 animals; 4 weeks treatment, n = 5 – 12 animals; 8 weeks treatment, n = 6 – 8 animals. The means are derived from highly variable data, sd was approximately 50 – 150% of the mean in many cases. Numbers in **bold** are statistical differences from controls as determined by Dunnett's test at $\alpha = 0.05$.

Table 4.10.3.1.Study 7.4 (DAR Table 6.5.4.1-4): Mean serum hormone levels in both male strains from terminal samples from the 4- and 8-week necropsy time points.								
Dose	**F344**				**SD**			
ppm	**0**	**25**	**100**	**500**	**0**	**25**	**100**	**500**
4 weeks								
Prl	39.42	57.60	57.05	45.72	59.98	42.54	49.82	32.49
T	1.17	2.79	2.58	3.27	6.07	5.57	9.35	6.15
LH	4.41	4.18	4.47	4.49	4.06	2.00	2.10	2.57
8 weeks								
Prl	70.91	60.54	53.67	71.87	35.28	34.01	40.97	33.23
T	2.56	2.42	2.56	2.68	3.01	3.41	4.28	3.79
LH	4.37	4.64	3.84	4.75	1.86	1.71	2.50	2.32

Prl = prolactin, ng/ml; T = testosterone, ng/g; LH = luteinizing hormone, ng/ml. 4 weeks treatment, n = 4 animals; 8 weeks treatment, n = 7 – 8 animals. The means are derived from highly variable data, sd was approximately 50% of the mean in many cases with a few instances of similar magnitude to the mean. There were no statistical differences from controls.

These hormone level data provide some weak support for MoA #9 (dopamine agonism/enhancement, table 6.5.4.1-5) with the key signature of a decrease in Prl levels, which would only be observed with MoA #9 and not associated with other possible mechanisms outlined above leading to Leydig cell tumours (LCT). The values obtained were quite variable and mostly non significant. General trends can be seen in the data which support the decrease in Prl levels but only at the 4 week time point and not in the terminal samples from this time point. A compensatory increase in LH may act as the primary trophic stimulus over the two-year Fischer rat carcinogenicity study leading to LCT promotion. The LH data shows some increase at the 4 and 8 week time points in both the in-life and terminal bleeds but it is not conclusive. The additional concordance of a slight increase in T with increased LH levels in Fischer rats at 4-weeks is not strong enough data to support that this

LH increase is biologically meaningful. Due to the persistent compensatory nature of the hypothalamic-pituitary-gonadal (HPG) axis, the pulsatile nature of gonadotropin release coupled with the fact that chronic two-years of sulfoxaflor exposure was required for increased LCT size in Fischer rats, it is not surprising that the changes observed in the hormone data are temporal in nature. Leydig cell effects in the guideline toxicity studies occurred only at the two-year time point but the high background incidence of this tumour type in the F344 rat makes any interpretation of the cause of treatment related increases in incidence unclear. There was no concomitant positive control treatment using dopamine agonists such as ergot alkaloids that can profoundly inhibit prolactin secretion in vivo. Use of such agents may have provided clearer support for the hypothesised MoA.

Table 4.10.3.1.Study 7.5 (DAR Table 6.5.4.1-5): Summary of Hormone Panel Data

Strain	Hormone	2wk	4 wk	4 wk Terminal	8 wk	8 wk Terminal
F344	LH:	---	↑	---	---	---
	Prl:	---	↓	---	---	---
	T:	---	---	↑	---	---
Crl:CD(SD)	LH:	↑	---	---	---	↑
	Prl:	---	↓	↓	---	---
	T:	↑	---	---	---	---

--- indicates no dose-dependent change; ↑ and ↓ indicate a dose-dependent increase or decrease, respectively.

If #7 (biliary elimination of testosterone) or #6 (reduced testosterone biosynthesis) were occurring there would be a dose-dependent decrease in testosterone levels, which was not seen at any time point in either strain. However, the results presented above only provide data for serum levels of testosterone. Numerous public domain and published reports indicate that testicular testosterone measurements would be a more sensitive endpoint for perturbations in testosterone production, often a secondary consequence of for example, bromocriptine-mediated reductions in prolactin secretion leading to hypoprolactinaemia in the rat.

Sacrifice and Pathology

1. Organ weights:

There was a treatment-related increase in liver weights of rats given 500ppm sulfoxaflor in both strains at 4-weeks and in Fischer rats at 8-weeks (table 6.5.4.1-6). There were no treatment-related effects on liver weights at 25 or 100ppm, nor testis weights at any dose level tested.

Table 4.10.3.1.Study 7.6 (DAR Table Table 6.5.4.1-6): Organ weights summary in both male strains from terminal samples from the 4- and 8-week necropsy time points.

Dose ppm	F344				SD			
	0	**25**	**100**	**500**	**0**	**25**	**100**	**500**
4 weeks								
Abs Liver (g)	10.15	10.97	11.04	12.41	17.57	19.69	17.83	21.01
	3.58	3.78	3.88	**4.30**	3.84	4.02	3.91	**4.37**
Rel Liver	2.50	2.72	2.62	2.64	3.47	3.49	3.20	3.40
Abs Test (g)	0.89	0.94	0.93	0.92	0.76	0.71	0.70	0.71
Rel Test								

Table 4.10.3.1.Study 7.6 (DAR Table Table 6.5.4.1-6): Organ weights summary in both male strains from terminal samples from the 4- and 8-week necropsy time points.								
8 weeks								
Abs Liver (g)	10.75	11.14	11.65	**12.37**	20.70	18.92	19.28	19.92
	3.54	3.53	3.68	**3.92**	3.71	3.66	3.66	3.84
Rel Liver	2.92	2.78	2.69	3.05	3.71	3.76	3.76	3.60
Abs Test (g)	0.96	0.88	0.86	0.97	0.67	0.73	0.72	0.70
Rel Test								

Rel Liver, g/100g; Test = testes, Rel Test g/100g; 4 weeks treatment, n = 4 animals; 8 weeks treatment, n = 7 – 8 animals. Numbers in **bold** are statistical differences from controls as determined by Dunnett's test at $\alpha = 0.05$.

2. Immunohistochemistry:

In the investigation of MoA #9, immunohistochemistry (IHC) of luteinizing hormone receptor (LHR) was performed on Fischer rat testes at the 4- and 8-week necropsy time points. As the largest hormone changes were observed at the 4-week time point, these IHC slides were blinded and quantified for number of Leydig cells with intracellular staining of LHR out of 1000 Leydig cells counted in two cross-sections of tissue. There was no treatment-related effect on the percentage of Leydig cells with cytoplasmic staining.

Targeted gene expression

1. Rat testicular LH receptor and Prl receptor:

Real-time PCR was performed on 4- and 8-week isolated Fischer rat testis mRNA for the LH receptor (*LHR*) and prolactin receptor (*PrlR*) genes in order to determine if there was molecular concordance to the hormone data, which supported MoA #9 (dopamine agonism/enhancement). In the MoA #9 pathway, dopamine agonism leads to decreased Prl release from the anterior pituitary gland thereby lowering circulating blood Prl levels. In rats, but not humans, Leydig cell prolactin levels are involved in regulation of *LHR* gene expression. Lower *LHR* expression would lead to a transient dip in testosterone production, leading to HPG-axis feedback stimulation and ultimately to increased LH release. Therefore if MoA #9 were operant, *LHR* gene expression would be decreased consistent with decreased circulating Prl hormone and increased LH.

Consistent with MoA #9 (and in agreement with the hormone panel data at 4 weeks), there was a ~2-fold dose-dependent decrease in *LHR* gene expression at the 4-week, but not 8-week, time point (table 6.5.4.1-7). In addition, there was a decrease in *PrlR* gene expression as well at the 4-week, but not 8-week, time point. While not statistically significant the magnitude of gene expression changes is consistent with the dynamic range of these genes *in vivo* and likely represents a biologically meaningful effect based on alterations in hormone levels. This conclusion is supported by a recent publication where administration of exogenous Prl to rats for 4-weeks resulted in ~2-fold increase in *LHR* gene expression.

Table 4.10.3.1.Study 7.7 (DAR Table 6.5.3.5-7): Targeted gene expression – rat testicular LH receptor and Prl receptor.

XDE-208 4-week Treatment-F344 Rats				
Dose/Gene	**LHR**	**PrlR**	**SD (LHR)**	**SD (PrlR)**
Control	**1**	**1**	0.27	0.21
25 ppm	**0.94**	**0.79**	0.35	0.34
100 ppm	**0.91**	**0.97**	0.11	0.26
500 ppm	**0.64**	**0.62**	0.12	0.18
XDE-208 8-week Treatment-F344 Rats				
Dose/Gene	**LHR**	**PrlR**	**SD (LHR)**	**SD (PrlR)**
Control	**1**	**1**	0.22	0.07
25 ppm	**0.78**	**0.79**	0.14	0.25
100 ppm	**1.19**	**1.33**	0.18	0.12
500 ppm	**1.12**	**1.08**	0.08	0.10

2. Steroidogenic Genes – MoA #6: (Reduced testosterone biosynthesis):

Reduced testosterone biosynthesis (MoA #6) is thought to be of low plausibility due to the fact that there was no effect in any study on female reproductive parameters, which would have been expected with this MoA as androgens are the precursors to estrogens; however, there was an effect of sulfoxaflor on serum cholesterol, which is the starting molecule used in the steroidogenic pathway, as well as a slight delay in preputial separation. As testis mRNA was available from the LHR and PrlR evaluations, gene expression on a suite of steroidogenic enzymes was also performed as an indicator of potential testosterone synthetic capability and an indirect indicator of potential testicular testosterone concentration.

There was no dose-dependent effect of treatment on any measured gene in the steroidogenic pathway including *StAR* (steroidogenic acute regulatory protein), *Cyp11a1* (P450side chain cleavage), *Cyp17a1* (17α-hydroxylase), *HSD3b* (3β hydroxysteroid dehydrogenase), or *SDR5a1* (5-α reductase). If reduced testosterone biosynthesis were the operant MoA, one or more of these genes would be affected. Furthermore, the hormone panel data would have shown a transient decrease in circulating levels of testosterone, which was not observed in either strain at any time point. Taken together these data, as well as a lack of female reproductive effects, refute decreased steroidogenesis (MoA #6) as the operant MoA.

Conclusions

This study was designed to investigate the MoA for the increased size of Fischer rat LCTs observed in the sulfoxaflor 2-year rat oncogenicity study at 100 and 500ppm, and increased incidence of bilateral LCTs at 500ppm. The effect in question is subtle in nature and the background incidence of Fischer rat LCTs is 75-100% in 2-year studies compared to 1-5% in CD rats, even less in CD-1 mice, and orders of magnitude lower in ranges of 0.01 – 0.00004% for humans. These interspecies differences in background incidence are well understood, and result from quantitative and qualitative differences in the Leydig cell response to hormonal stimuli. Rat Leydig cells contain >10-fold more LH receptors than humans, which confers greater sensitivity to slight changes in circulating LH levels. In addition to this quantitative

difference, rat, but not human, Leydig cells have both PrlR and GnRH receptors (GnRHR) on their surface.

In mammals, lactotrophs located in the anterior pituitary gland, are "spontaneous" secretors of prolactin, requiring no acute stimulatory input to achieve this action. Indeed, a continuous basal level secretion of copious amounts of prolactin would be the norm if it were not for the inhibitory regulatory input of dopamine acting on the D2 receptors found on the surface of the lactotrophs. Thus, the major regulatory input to mammalian lactotrophs is inhibitory. This inhibition of prolactin secretion is mediated almost entirely by dopamine produced by the tuberoinfundibular neurones in the arcuate nucleus of the hypothalamus. This dopamine is released from nerve terminals at the median eminence into the hypothalamic-hypophysial portal vessels. The portal veins run from the median eminence along the pituitary stalk, conveying dopamine (as well as other controlling hormones) to the capillary bed in the anterior lobe of the pituitary gland. Dopamine acts at several levels of cell function - not only inhibiting acute release of prolactin from lactotrophs but also inhibiting transcription of the hormone and acts as a potent antimitotic / antiproliferative factor in pituitary lactotrophs. Mice with a disrupted D2 dopamine receptor gene have chronic hyperprolactinaemia and develop anterior lobe lactotroph hyperplasia (*Kelly et al., 1997*).

Stimulation of rat Leydig cells through both PrlR and GnRHR are a rat-specific mechanism by which LCT formation can occur. For PrlR involvement in LCT, dopamine agonists such as ergot derivatives (e.g., mesulergine) reduce Prl release by the anterior pituitary gland. This results in decreased binding of Prl to PrlR on Leydig cells, and is thought to lead to down regulation of the LH receptor and transient reductions in testosterone production, which feeds back to induce LH release from the pituitary leading to Leydig cell stimulation and hyperplasia over time. Normal regulation of gonadotropin (LH and FSH) release in mammals is complex involving many inter-relating pathways, feedback loops and chemical mediators. For instance feedback inhibition by prolactin itself on GnRH release from the hypothalamus is thought to occur and this could have repercussions with respect to LH release with declining levels of circulating prolactin.

As outlined initially in this report, there are nine known modes-of-action for developing rodent Leydig cell tumours. This report presents the plausibility of these alternative modes of action (MoA) in the context of the sulfoxaflor-induced effect in Fischer rat Leydig cell tumours. Before these findings were observed at the end of the rat chronic/carcinogenicity study, the only related effects were limited to a possible earlier onset of LCTs at the one-year time point (as determined from comparison with concurrent controls), that was within the historical control range (0, 1, 3, and 3 LCT incidences were seen in the 0, 25, 100, and 500ppm groups in F344 rats from the long-term / carcinogenicity study; historical control incidence was 0-3) and a marginal 2.4-day delay in preputial separation at 400ppm in the two-generation study in Crl:CD(SD) rats. There were no other effects on any other reproduction end point from other studies, including: testes, epididymides, or accessory glands in F344 rats, CD rats or CD-1 mice; sperm parameters (counts, motility, morphology); reproduction – fertility, mating indices, time to mating; development, including developmental neurotoxicity; markers of androgenic/anti-androgenic effects; and, male anogenital distance. The lack of any other reproductive findings in the suite of toxicity studies performed with sulfoxaflor were used as the basis for this dopamine agonist MoA study.

The available data about rat Leydig cells and the differences in physiological hormonal control in different species such as rats and humans suggest that human Leydig cells are quantitatively less sensitive than rat Leydig cells in their proliferative response to LH, and

hence in their sensitivity to chemically induced LCTs. Many researchers accept that nongenotoxic compounds that induce LCTs in rats most likely have low to zero relevance in humans. The finding of increased incidences of LCTs in the F344 rat used in the combined chronic toxicity / carcinogenicity study has presented difficulties. The interpretation of results from using this strain is controversial, owing to the difficulty in distinguishing chemically-induced tumours from age-dependent background tumours.

Results from the biliary elimination portion of this study revealed no treatment-related differences in the mean ^{14}C-testosterone derived radioactivity excreted in the bile, levels in circulating plasma, or in bile flow for Crl:CD(SD) and Fischer rats. Taken together, these data refute MoA #7 (biliary elimination of testosterone) as the operant MoA.

Results from the serum hormone panel portion of the study are equivocal; mean values are associated with highly variable individual results which may serve to mask subtle effects. An ~2-fold dose-dependent increase in LH concentrations concomitant with a ~1.7-fold dose-dependent decrease in Prl levels for Fischer rats was seen at the 4-week time point. This increase in serum LH was accompanied by an ~3-fold dose-dependent increase in T at 4 weeks. A similar hormone pattern was seen in Crl:CD(SD) rats, albeit with a different timing, where an increase in LH and concomitant increase in T occurred at 2-weeks, and a decrease in Prl occurred at 4-weeks. There was no effect of treatment on Prl, LH, or T at all other time points in Fischer (2- and 8-weeks) and Crl:CD(SD) (8-weeks) rats.

Consistent with MoA #9 and the decreased Prl levels in the 4-week Fischer rat hormone data, there was an ~2-fold dose-dependent decrease in LHR gene expression at the 4-week, but not 8-week, time point. In addition, there was a decrease in PrlR gene expression at the 4-week, but not 8-week, time point. While not statistically significant, the magnitude of gene expression changes is consistent with the dynamic range of these genes *in vivo* and likely represents a biologically meaningful effect based on alterations in hormone levels.

There was no dose-dependent effect of treatment on any measured gene in the steroidogenic pathway including *StAR, Cyp11a1, Cyp17a1, HSD3b, or SDR5a1*. If reduced testosterone biosynthesis was the operant MoA, one or more of these genes would have been affected. It is speculative whether the hormone panel data would have shown a transient decrease in circulating levels of testosterone, (no changes were observed in either strain at any time point), and testicular levels of testosterone have been shown to be reduced with dopamine agonist treatment (*Suescun et al., 1985*).

Dopamine agonism could very well operate through sulfoxaflor-mediated enhancement of dopamine release, potentially though agonism of α4β2 or α4α6β2 central nicotinic acetylcholine receptors (nAChRs), which are known to play a key regulatory role in dopamine release from dopaminergic neurons in the brain. As mentioned previously, sulfoxaflor is an agonist to the foetal rat nicotinic acetylcholine receptor (nAChR) and the insect neuronal nAChR is the target of the insecticidal mechanism for sulfoxaflor. Therefore it should come as no surprise that sulfoxaflor could display weak agonistic activity against mammalian central nicotinic receptors. It is a well known effect that in the chronically treated rat, focal Leydig cell hyperplasia and Leydig cell tumours can be readily induced by a wide range of chemically diverse drugs and chemicals, including dopamine agonists such as mesulergine, dopamine reuptake inhibitors like oxolinic acid, antiandrogens like flutamide, LHRH analogues such as leuprolide acetate and goserelin, peroxisome proliferators, and histamine receptor antagonists like cimetidine (*Dirami et al., 1996; 1998; Prentice et al., 1992*). The common proposed mechanism of action for these various classes of compounds is through

interference with Leydig cell control mechanisms at a variety of points along the hypothalamic/pituitary/testicular axis. Low prolactin levels have been implicated in the development of Leydig cell tumours, while chronic hyperprolactinaemia is known to reduce the incidence of Leydig cell tumours in rats (*Bartke et al., 1985*). Leydig cell tumour formation in rats is attributed to increased LH levels, a condition that favours Leydig cell hyperplasia (*Prentice et al., 1992; Yamada et al., 1994*).

It can be argued there are some deficiencies in the present study. For instance, why were no positive controls run concurrent with the treatment groups. There is an abundance of published reports where researchers have used dopamine agonists such as the ergot derivatives to investigate changes in serum Prl and rat Leydig cell LH/hCG receptor binding sites (*Dirami et al., 1996, 1998; Waeber et al., 1983; Suescun et al., 1985; Prentice et al., 1992*) and confirmed the development of Leydig cell hyperplasia and increased incidences of LCTs. Such a positive control might have aided in the interpretation of the hormonal panel results and at the very least provided a set of results to indicate expected outcomes (for example, after approximately 5 weeks of treatment, mesulergine causes hypertrophy of Leydig cells that is followed by hyperplasia – *Dirami et al., 1996*).

A hypothesis was presented based on altered hormonal regulation that is plausible and describes results that provide evidence supporting MoA #9 in the form of decreased circulating Prl levels, with increases in LH and T levels, along with decreases in testis LHR gene expression. In the case of sulfoxaflor, the data presented in this study is equivocal with respect to the measured hormonal levels. In addition, the measurement of testicular testosterone might have been a more sensitive indicator of hormonal perturbation than serum testosterone. The results for the testes LHR and PrlR gene expression and investigation into steroidogenic enzyme expression were supportive of the proposed MoA. Decreases in the LH binding capacity of Leydig cells (due to a decrease in the number of receptors) have been reported as a consequence of dopamine agonist exposure in male rats (*Dirami & Cooke, 1998; Waeber et al., 1983*) as well as changes in the production of testosterone upon challenge of the Leydig cells with hCG or LH.

Based on the data presented in this study, it is plausible though not conclusive that the LCT promotion seen in the rat chronic/carcinogenicity study was through weak, but chronic, enhancement of dopamine release, and the subsequent inhibition of prolactin release from the pituitary gland, ultimately leading to a dopamine agonism/enhancement LCT MoA in a uniquely susceptible animal model, the Fischer 344 rat. This MoA would be considered to have no relevance to humans, per se. In addition to providing data to support or refute specific LCT MoA, the observation of hormone level alterations in this study are equivocal with respect to supporting a hormonally-mediated, and thereby threshold, nonlinear mode-of-action.

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Study 8: Proof of Concept Study: Dopamine microdialysis experiment. DAR Section B.6.5.4.2.

Sulfoxaflor is postulated to increase dopaminergic neurotransmission in the rat tuberoinfundibular (TIDA) system. In turn, the increased outflow of dopamine into the hypothalamic portal circulation inhibits the release of prolactin by the pituitary. This hypothesis was tested by measuring the effects of reverse dialysis of sulfoxaflor on the extracellular concentration of dopamine in the mediobasal hypothalamus (section B.6.5.4.2; Rowley and Heal, 2011). The concentration of analytes crossing the semi-permeable membrane of the microdialysis probe was assumed to be about 10-fold lower than the concentration present in the perfusion fluid, thus sulfoxaflor was reverse dialysed at a concentration of 400µM (to replicate a concentration of 40µM in the extracellular fluid of the mediobasal hypothalamus) and at the higher concentration of 2mM (to replicate a

concentration of ~200µM in the extracellular fluid of the mediobasal hypothalamus). In addition to measuring the effect of sulfoxaflor on dopamine efflux, the extracellular concentrations of its two major metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were also determined. A depolarising pulse of K+ ions produced a transient and sharply delineated increase in dopamine efflux indicating that these hypothalamic dopaminergic neurones were viable and normally responsive. Sulfoxaflor (400µM and 2mM) produced increases in the extracellular level of dopamine in the mediobasal hypothalamus. Sulfoxaflor and K+ ions increased the extracellular concentration of dopamine and produced concomitant reductions in the concentration of HVA; neither sulfoxaflor nor K+ ions altered the extracellular concentration of DOPAC. The data support the hypothesis that through its nAChR partial agonist properties sulfoxaflor increases dopamine efflux from TIDA neurones in the median eminence, and in turn, this effect is predicted to result in a decrease of prolactin secretion from the pituitary gland in the rat.

Report: Rowley H. L. And Heal, D. J. (2011). Effects of sulfoxaflor infusion on hypothalamic dopamine, DOPAC and HVA efflux – a microdialysis experiment in freely moving rats. RenaSci Consultancy Ltd, BioCity Nottingham, Pennyfoot Street, Nottingham, NG1 1GF, UK. Unpublished.

Report No.: DR-0404-3134-124; Study ID: RS867.

Dates: 2011

Guidelines: Non-guideline.

GLP: No. Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were not provided.

Deviations: This is an acceptable though non-guideline study, it is considered supplementary to the long-term chronic / carcinogenicity studies and suitable for a MoA investigation.

Deficiencies: Yes. An extended variation of this study could have also easily investigated dopamine agonists and/or reuptake inhibitors as supplemental positive controls in addition to K^+ spiking that may more closely mimic the proposed *in vivo* effects of sulfoxaflor. In addition, more time should have been allowed in between infusion events to allow dopamine responses to return to near baseline levels.

Executive Summary: Sulfoxaflor is a nicotinic acetylcholine receptor (nAChR) partial/weak agonist in the rat that is postulated to increase dopaminergic neurotransmission in the tuberoinfundibular (TIDA) system. In turn, the increased release of dopamine (DA) into the hypothalamic portal circulation further inhibits the release of prolactin by the pituitary. This hypothesis was tested by measuring the effects of reverse dialysis of sulfoxaflor on the extracellular concentration of DA in the mediobasal hypothalamus of male SD rats (n = 7). Since the concentration of analytes crossing the semi permeable membrane of the microdialysis probe is approximately 10 fold lower than the concentration present in the perfusion fluid (assumed, not measured), sulfoxaflor was reverse dialysed at a concentration of 400µM in the external, artificial cerebrospinal fluid (to replicate a concentration of 40µM in the extracellular fluid of the mediobasal hypothalamus) and at the higher concentration of 2mM (to replicate a concentration of approximately 200µM in the extracellular fluid of the mediobasal hypothalamus). In addition to measuring the effect of sulfoxaflor on DA release, the extracellular concentrations of its two major metabolites dihydroxyphenylacetic acid

(DOPAC) and homovanillic acid (HVA) were also determined. A depolarising pulse of 50mM K^+ ions was used as a positive control and enhancer of increased local dopaminergic activity. This pulse of K^+ ions produced a transient and sharply delineated increase in DA efflux confirming that these hypothalamic dopaminergic neurones were viable and normally responsive.

Sulfoxaflor (at external concentrations of 400µM and 2mM) produced dose related increases in the extracellular level of dopamine in the mediobasal hypothalamus. Relative to the initial baseline, the increases evoked by sulfoxaflor were 15.4% at 400µM and 25.8% at 2mM. Sulfoxaflor and K^+ ions increased the extracellular concentration of dopamine and produced concomitant reductions in the concentration of HVA; neither sulfoxaflor nor K^+ ions altered the extracellular concentration of DOPAC. The identical profiles of K^+ and sulfoxaflor indicate that sulfoxaflor was causing an increase in local, external dopamine concentrations from the hypothalamic dopaminergic neurones. Since a concentration of sulfoxaflor of 400µM in the dialysis perfusion fluid equates to an extracellular concentration of approximately 40µM, it is reasonable to hypothesise that *in vivo* a circulating concentration of ≥ 40µM sulfoxaflor would be capable of releasing DA from the TIDA neurones. Together, the data support the hypothesis that through its central nAChR agonist properties, sulfoxaflor increases DA efflux from TIDA neurones in the median eminence, and in turn, this effect is predicted to result in a decrease of prolactin secretion from the pituitary gland in the rat.

Materials and Methods

Materials:

1	**Test Material:**	Sulfoxaflor
	Synonyms:	XDE-208; (N-(Methyloxido(1-(6-(trifluoromethyl)-3-pyridinyl)ethyl)-λ^4-sulfanylidene)-cyanamide); [1-(6-Trifluoromethylpyridin-3-yl)ethyl](methyl)-oxido-λ^4-sulfanylidenecyanamide; Sulfoximine; X11422208; XR-208.
	Description:	White Solid
	Lot/Batch #:	Lot # E2162-34, TSN003725-001.
	Purity:	96.6% (w/w))
	CAS #:	946578-00-3
2	**Vehicle:**	LabDiet Certified Rodent diet #5002 (PMI Nutrition International, St. Louis, Missouri, US)
	Dose	Via continuous perfusion at a flow rate of 1.2µl/min into a centrally located microdialysis probe. 2 concentrations (400µM and 2mM) of sulfoxaflor were infused over a 40min period.
3	**Test Animals:**	
	Species:	Rat

Strain:	Sprague Dawley
Age/weight at study initiation:	8-10 weeks / 0.250 – 0.350 kg (all male).
Source:	Charles River Laboratories UK.
Housing:	Animals were house 4 per cage. Following surgery, animals were individually housed in dialysis bowls (dimensions 450mm internal diameter, 320mm wall height) with the microdialysis probe connected to a liquid swivel and a counter balanced arm to allow unrestricted movement.
Feed and Water:	Feed (Harlan Teklad 2018 Rodent Diet) and water (UK mains quality) were available *ad libitum*.
Environmental conditions:	**Temperature:** 21 ± 2 C **Humidity:** 55 ± 20% **Air Changes:** 15-20 times/hour **Photoperiod:** 12-hour light/dark
Acclimation period:	

Study Design:

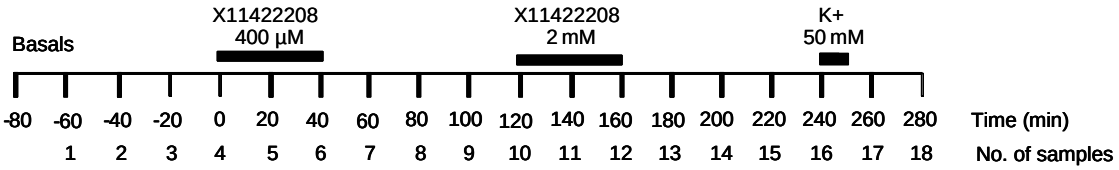
In life dates: 4th August to 15th August 2011.

Animal assignment and treatment groups: Rats from the Sprague Dawley strain were selected in preference over Fischer 344 rats because the SD strain is the most frequently used in microdialysis experiments. Extensive historical data is available on this strain from the CRO against which results can be compared. In addition, the stereotaxic coordinates used to place probes in the mediobasal hypothalamus are only valid for SD rats of 250 – 350g.

Rats were anaesthetised with isoflurane (5% to induce, 2% to maintain) in O_2 (1L/min) delivered via an anaesthetic unit (Burtons Medical Equipment Ltd, UK). A single probe study was performed whereby a concentric microdialysis probe with an exposed polyarylethersulphone (PAES) membrane tip (CMA, Sweden) was stereotaxically implanted into the hypothalamus (2mm tip, coordinates: AP: -1.8 mm; L: +/ 0.8 mm relative to bregma; V: 9.2mm relative to the skull surface) of 8 male SD rats. Coordinates were taken from the stereotaxic atlas of *Paxinos and Watson* (1986). The upper incisor bar was set at 3.3 mm below the interaural line so that the skull surface between the principal skull landmarks of the bregma and lambda was horizontal. Additional burr holes were made for skull screws (stainless steel) and the probes were secured using dental cement.

Following surgery, animals were individually housed in dialysis bowls (dimensions 450mm internal diameter, 320mm wall height) with the microdialysis probe connected to a liquid swivel and a counter balanced arm to allow unrestricted movement. Rats were allowed a recovery period of at least 16hr with food and water available *ad libitum*. During this time the probes were continuously perfused at a flow rate of 1.2µl/min with an artificial cerebrospinal fluid (aCSF; Harvard Apparatus, UK) of the following electrolyte composition: Na^+ 150.0mM; K^+ 3.0mM; Mg^{2+} 0.8mM; Ca^{2+} 1.4mM; PO^{3-} 1.0mM; Cl^- 155.0mM.

Microdialysis and administration of drugs: Dialysate samples were collected from freely-moving rats at 20min intervals with four basal samples collected prior to the onset of infusion of sulfoxaflor. Two concentrations (400µM and 2mM), were each infused at 1.2µl/min in aCSF for 40 min as shown in the diagram below. Before termination of the experiment, high potassium (K^+, 50mM) was infused at 1.2µl/min in aCSF for 10min to act as a positive control.



Extracellular fluid samples were collected every 20min into Eppendorf vials (300µl) containing 0.1 M perchloric acid (5.0µl) to prevent oxidation of DA and its metabolites and frozen in dry ice. After the completion of the experiment, all samples were stored at -80ºC until analysis by HPLC with electrochemical detection which was conducted over the remainder of the week following experimentation.

Statistics: All data were log transformed. Data were analysed twice, once using the data from each time point, then using the average data from - 60 to 0 min, 0 to 120 min, 120 to 240 min and 240 to 280 min. Analysis was by mixed linear model, using the first order autoregressive plus random effect correlation structure, with animal as the subject. For the analysis of each time point, there were fixed factors for treatment and the treatment by time interaction. For the analysis of averaged data, the only fixed factor was treatment. Sulfoxaflor at each time point was compared to baseline by separate Williams' tests (for example, 0 20min data and 120 to 140min data were included in the same Williams' test). K^+ at each time point was compared to baseline by the multiple t-test. A p value of < 0.05 was considered statistically significant. The n values reported in all tables and figures refer to the number of rats from which data was reported (7 animals, excluding 1 animal where the probes were incorrectly positioned).

Methods:

HPLC analysis: Detection and subsequent quantification of DA, DOPAC and HVA in the dialysis samples was based on reverse-phase, ion-pair HPLC coupled with electrochemical detection and involved the use of an ALEXYS® monoamine analyser (Antec Leyden, The Netherlands). The system consisted of two separate analytical columns (ALF-115, 150 mm x 1 mm internal diameter) that shared a dual loop autosampler allowing for one sample to be simultaneously analysed by two systems optimised for different neurotransmitters. In this instance, one column separated DOPAC and HVA while the other separated DA. Two solvent delivery pumps (LC 110) were used to circulate the respective mobile phases (DOPAC and HVA: 50mM citric acid, 50mM phosphoric acid, 8mM NaCl, 0.1mM EDTA, 3mM 1-octane sulphonic acid, 10% methanol, pH 3.25; DA: 50mM phosphoric acid, 8mM NaCl, 0.1mM EDTA, 2.5mM 1-octane sulphonic acid, 20% methanol, pH 6.0) at a flow rate of 50µl/min and an Antec in line degassing unit was used to remove air. Samples (10µl) were injected onto the columns via an autosampler (AS 110) with a cooling tray set at 4ºC. Antec DECADE II® electrochemical detectors were used and Antec micro VT 03 cells employing a high density, glassy carbon working electrode (+0.59 V for DOPAC and HVA, +0.3 V for DA) combined with an ISAAC reference electrode. The electrode signal was integrated using Antec's CLARITY® data acquisition system. Individual stock solutions of DA, DOPAC and

HVA (1.0mM) were prepared by their dissolution in a mixture of equal quantities of deionised water and 0.1M perchloric acid (in order to prevent oxidation) and stored at 4ºC. A working solution containing all the transmitters and metabolites was prepared daily by dilution in aCSF.

Histology: At the end of the experiment, rats were killed and their brains rapidly removed and stored in a 10% v/v formal saline solution for a minimum of 5 days. Probe placements were visualised and localised with reference to a stereotaxic atlas (*Paxinos and Watson, 1986*). Data are reported only from animals where probe membranes were correctly positioned (n=7). Rat 1 was excluded from the analysis as the probe location was found to be positioned slightly outside of the hypothalamus.

Drugs and reagents: Sulfoxaflor was supplied by Dow AgroSciences (Lot No. E2162-34, Batch No. TSN003725-001). For the infusion of 2mM, vehicle (aCSF) was added to the vial containing sulfoxaflor and vortexed for 20s then gently warmed under a hot tap for 10s. A clear solution was formed which was suitable for infusion (pH 7.1). The 400µM solution was prepared by dilution in aCSF from the stock solution (2mM). A clear solution was formed which was suitable for infusion (pH 7.0). Potassium chloride (Batch No. 0740210) was obtained from Fisher Scientific (UK). For infusion of 50mM vehicle (aCSF) was added to the vial containing potassium chloride and vortexed for 20s. A clear solution was formed which was suitable for infusion (pH 7.0 7.1).

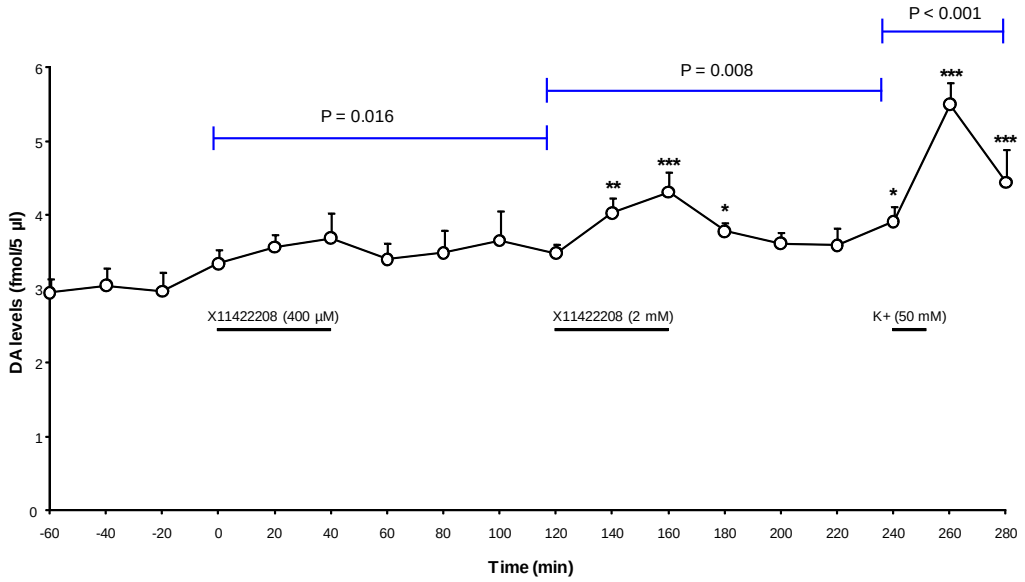
All reagents used in HPLC analysis were of HPLC grade. Phosphoric acid, EDTA, methanol, 1 octane sulphonic acid, perchloric acid, citric acid, sodium chloride and 10% formal saline solution were obtained from Fisher Scientific (UK). Dopamine hydrochloride (Batch No. 1381581), 3,4 dihydroxyphenylacetic acid (Batch No 1364209) and homovanillic acid (Batch No 107K5002) were purchased from Sigma Aldrich (UK).

Results and Discussion

Effects of sulfoxaflor infusion on extracellular levels of dopamine in the rat hypothalamus

Sulfoxaflor at the lower dose (400µM) had a small effect on the extracellular dopamine levels in the rat hypothalamus (figure 6.5.4.2-1 and table 6.5.4.2-1). Dopamine efflux averaged over the duration of the sulfoxaflor (400µM) infusion was statistically significantly ($p = 0.016$) increased (table 6.5.4.2-2) over baseline (15.4%). At the higher concentration, sulfoxaflor (2mM) resulted in a significant increase in extracellular dopamine, peaking at about 40% (t=160min) after the second infusion ($p<0.001$), figure 6.5.4.2-1 and table 5.5.4.2-1. Dopamine efflux (25.8%) averaged over the duration of the higher dose infusion was also significantly ($p = 0.008$) increased (table 6.5.4.2-2). K^+ (50mM) increased dopamine levels with a maximum increase of 78 ± 17%, 20 min after onset of perfusion ($p<0.001$), figure 6.5.4.2-1 and table 6.5.4.2-1) and overall ($p<0.001$; table 6.5.4.2-2) had an average efflux 61% over the initial mean baseline value.

(a) Levels (fmol/5µl)



(b) Percentage of baseline

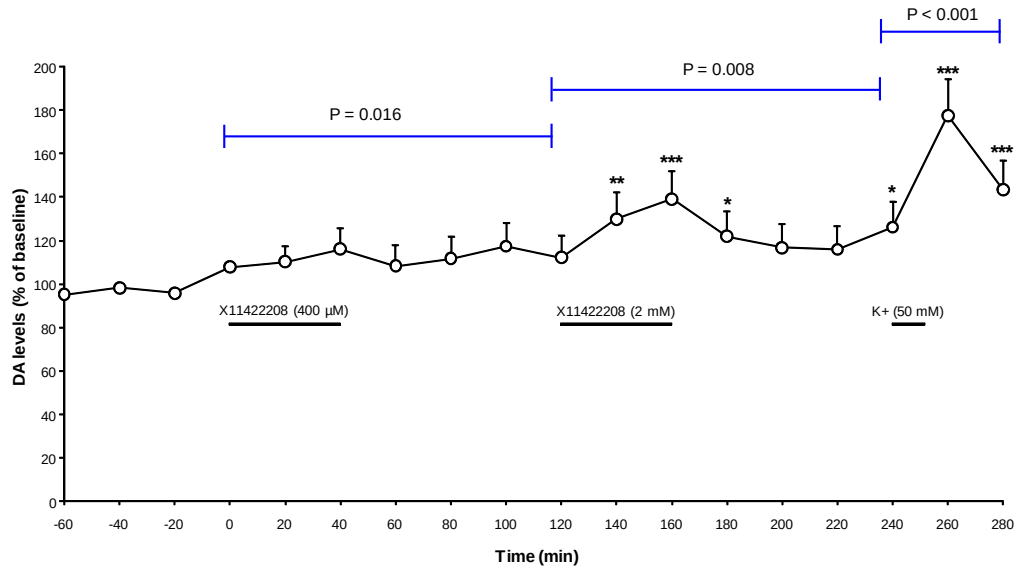


Figure 4.10.3.1.Study 8.1 (DAR Figure 6.5.4.2-1). Effects of sulfoxaflor infusion (400µM and 2mM) on extracellular levels of dopamine in the hypothalamus of the freely moving rat (a. levels and b. % of baseline). Results are adjusted means; n=7. SEMs are calculated from the residuals of the statistical model. Drug infusion is indicated by the horizontal bar. Data analysed by mixed linear model with animal as subject and treatment and treatment by time as factors followed by Williams' test (sulfoxaflor) and the multiple t-test (K^+). Significant differences versus baseline values are denoted by *p<0.05, **p<0.01 and ***p<0.001.

Table 4.10.3.1.Study 8.1 (DAR Table 6.5.4.2-1). Effects of sulfoxaflor infusion (400µM and 2mM) on extracellular levels of dopamine in the hypothalamus of the freely-moving rat (levels and % of baseline).

Time (min)	Concentration	Levels (fmol/5 µl)	% of baseline
-60		2.947 ± 0.189	95.17 ± 0.00
-40		3.043 ± 0.230	98.26 ± 0.00
-20		2.967 ± 0.254	95.81 ± 0.00
0		3.339 ± 0.191	107.84 ± 0.00
20	sulfoxaflor (400µM)	3.565 ± 0.164	110.29 ± 7.48
40		3.685 ± 0.347	116.12 ± 9.56
60		3.399 ± 0.221	108.24 ± 9.60
80		3.487 ± 0.305	111.73 ± 10.27
100		3.651 ± 0.397	117.39 ± 10.98
120		3.480 ± 0.123	112.09 ± 10.59
140	sulfoxaflor (2mM)	4.027 ± 0.204**	129.85 ± 12.34**
160		4.311 ± 0.268***	139.12 ± 13.26***
180		3.778 ± 0.121*	121.96 ± 11.64*
200		3.612 ± 0.150	116.63 ± 11.14
220		3.587 ± 0.230	115.83 ± 11.07
240		3.908 ± 0.204*	126.19 ± 12.07*
260	K^+ (50mM)	5.499 ± 0.293***	177.58 ± 16.98***
280		4.442 ± 0.447***	143.44 ± 13.72***

Data are means ± SEM. Means are the adjusted means from the mixed linear model with animal as the subject. SEM's are calculated from the residuals of the statistical model. % of baseline calculated from the adjusted means. P values for the comparison to baseline use Williams' test for sulfoxaflor and the multiple t-test for K^+. *p<0.05, **p<0.01, ***p<0.001 significantly different from baseline.

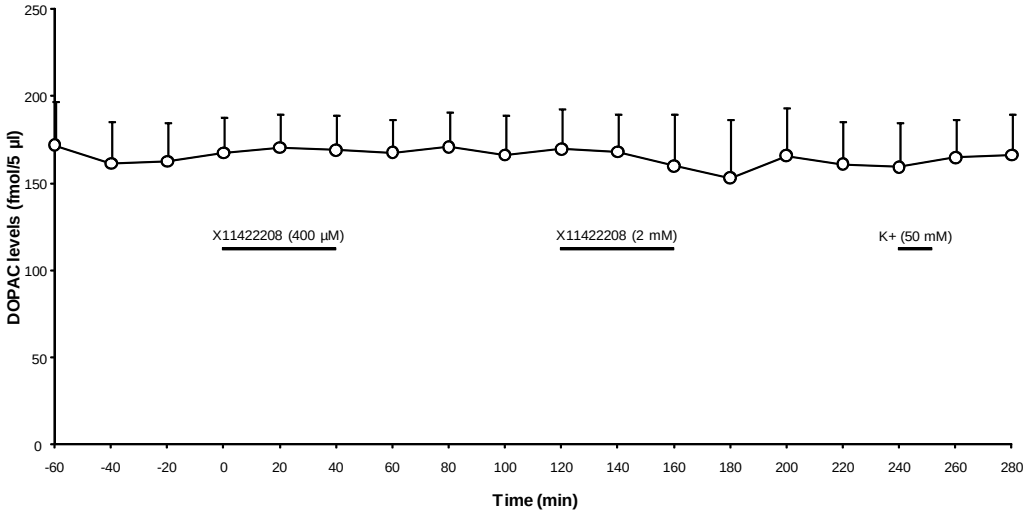
Table 4.10.3.1.Study 8.2 (DAR Table 6.5.4.2-2). Average results (fmol/5µl) in the hypothalamus.

Treatment	n	Mean	SEM	% of baseline		p vs
				Mean	SE	baseline

Dopamine						
Baseline	7	3.07	1.44			
sulfoxaflor 400µM	7	3.54	1.64	115.4	6.2	0.016*
sulfoxaflor 2mM	7	3.86	1.74	125.8	9.5	0.008**
K^+	7	4.94	2.19	161.0	14.9	<0.001***
DOPAC						
Baseline	7	166	33			
sulfoxaflor 400µM	7	169	31	101.9	6.3	1.000
sulfoxaflor 2mM	7	161	36	97.1	8.3	0.877
K^+	7	165	33	99.8	10.4	0.987
HVA						
Baseline	7	59.9	8.8			
sulfoxaflor 400µM	7	57.3	8.7	95.7	6.0	0.571
sulfoxaflor 2mM	7	56.2	10.4	93.9	8.2	0.571
K^+	7	47.3	9.8	78.9	8.3	0.037*

Means are the adjusted means from the mixed linear model with animal as the subject. SEM's are calculated from the residuals of the statistical model. % of baseline calculated from the adjusted means. P values for the comparison to baseline use Williams' test. *p<0.05, **p<0.01, ***p<0.001

(a) Levels (fmol/5µl)



(b) Percentage of baseline

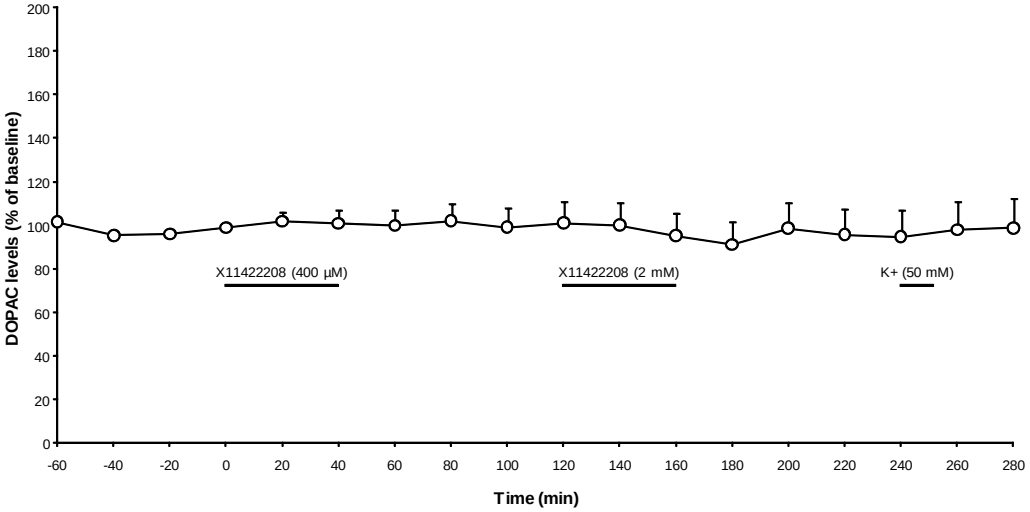


Figure 4.10.3.1.Study 8.2 (DAR Figure 6.5.4.2-2). Effects of sulfoxaflor infusion (400µM and 2mM) on extracellular levels of DOPAC in the hypothalamus of the freely moving rat (a. levels and b. % of baseline). Results are adjusted means; n=7. SEMs are calculated from the residuals of the statistical model. Drug infusion is indicated by the horizontal bar. Data analysed by mixed linear model with animal as subject and treatment and treatment by time as factors followed by Williams' test (sulfoxaflor) and the multiple t-test (K^+). No significant differences versus baseline values.

Table 4.10.3.1.Study 8.3 (DAR Table 6.5.4.2-3). Effects of sulfoxaflor infusion (400µM and 2mM) on extracellular levels of DOPAC in the hypothalamus of the freely-moving rat (levels and % of baseline).

Time (min)	Concentration	Levels (fmol/5 µl)	% of baseline
-60		171.846 ± 25.175	101.46 ± 0.00
-40		161.366 ± 24.239	95.27 ± 0.00
-20		162.536 ± 21.888	95.96 ± 0.00
0		167.468 ± 20.577	98.88 ± 0.00
20	sulfoxaflor (400µM)	170.444 ± 19.009	101.74 ± 4.16
40		169.065 ± 20.075	100.87 ± 5.78
60		167.485 ± 19.127	99.89 ± 6.94
80		170.878 ± 20.005	101.88 ± 8.10
100		166.075 ± 22.895	98.98 ± 8.72
120		169.594 ± 23.262	101.05 ± 9.66
140	sulfoxaflor (2mM)	167.953 ± 21.879	100.03 ± 10.24
160		159.790 ± 30.070	95.14 ± 10.31
180		153.069 ± 33.516	91.11 ± 10.38
200		165.702 ± 27.444	98.61 ± 11.74
220		160.788 ± 24.238	95.65 ± 11.84
240		159.217 ± 25.420	94.69 ± 12.13
260	K^+ (50mM)	164.772 ± 21.915	97.97 ± 12.95
280		166.154 ± 23.572	98.77 ± 13.44

Data are means ± SEM. Means are the adjusted means from the mixed linear model with animal as the subject. SEM's are calculated from the residuals of the statistical model. % of baseline calculated from the adjusted means. P values for the comparison to baseline use Williams' test for sulfoxaflor and the multiple t-test for K^+. Not significantly different from baseline.

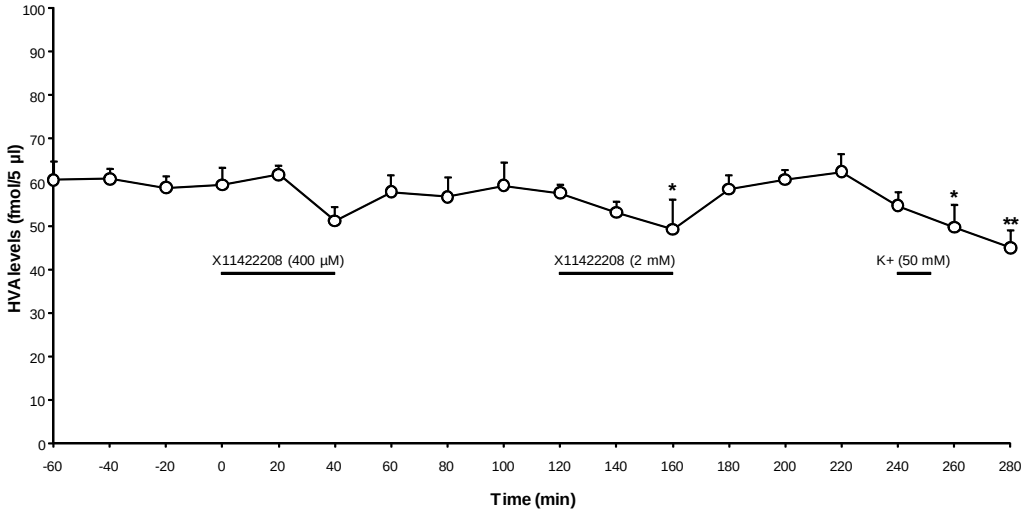
Effects of sulfoxaflor infusion (400µM and 2mM) on extracellular levels of DOPAC in the hypothalamus of the freely-moving rat

The effects of sulfoxaflor (400µM and 2mM) on extracellular levels of DOPAC in the hypothalamus are shown in figure 6.5.4.2-2 and table 6.5.4.2-3. Neither sulfoxaflor nor K^+ had any effect on DOPAC levels in the rat hypothalamus.

Effects of sulfoxaflor infusion on extracellular levels of HVA in the hypothalamus of the rat

The effects of sulfoxaflor (400µM and 2mM) on extracellular levels of HVA in the hypothalamus are shown in figure 6.5.4.2-3 and table 6.5.4.2-4. Sulfoxaflor (400µM) had no effect on HVA levels in the rat hypothalamus but the higher concentration (2mM) resulted in a significant decrease in extracellular HVA with levels falling to 82% of basal values, a change of 18 ± 7%, 40 min after the onset of infusion ($p<0.05$). Elevated K^+ also decreased HVA levels, with a maximum reduction of 25 ± 6%, 40 min after onset of perfusion ($p<0.01$).

(a) Levels (fmol/5µl)



(b) Percentage of baseline

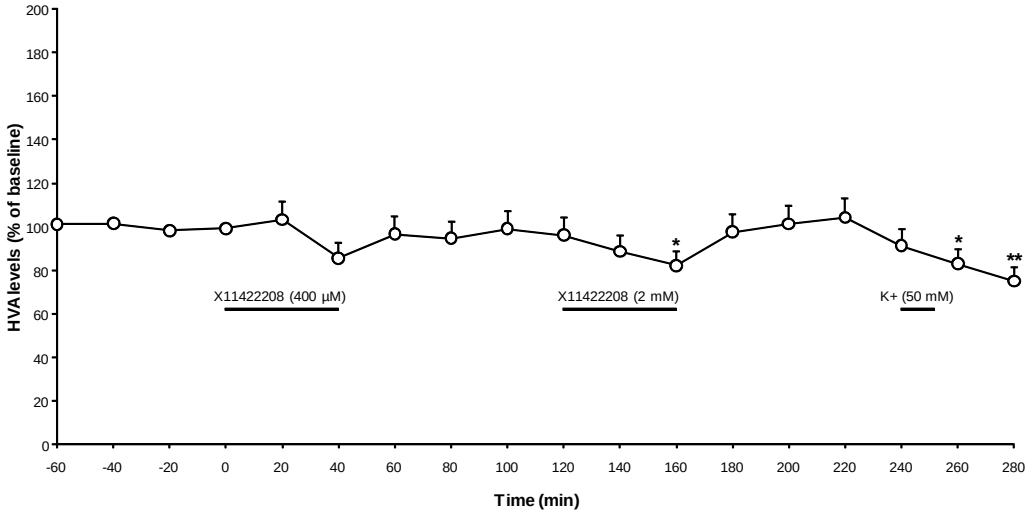


Figure 4.10.3.1.Study 8.3 (DAR Figure 6.5.4.2-3). Effects of sulfoxaflor infusion (400µM and 2mM) on extracellular levels of HVA in the hypothalamus of the rat (a. levels and b. % of baseline). Results are adjusted means; n=7. SEMs are calculated from the residuals of the statistical model. Drug infusion is indicated by the horizontal bar. Data analysed by mixed linear model with animal as subject and treatment and treatment by time as factors followed by Williams' test (sulfoxaflor) and the multiple t-test (K^+). Significant differences versus baseline values are denoted by *p<0.05, **p<0.01.

Table 4.10.3.1.Study 8.4 (DAR Table 6.5.4.2-4). Effects of sulfoxaflor infusion (400µM and 2mM) on extracellular levels of HVA in the hypothalamus of the freely-moving rat (levels and % of baseline).

Time (min)	Concentration	Levels (fmol/5 µl)	% of baseline
-60		60.527 ± 4.274	101.11 ± 0.00
-40		60.765 ± 2.427	101.51 ± 0.00
-20		58.754 ± 2.704	98.15 ± 0.00
0		59.391 ± 3.928	99.21 ± 0.00
20	sulfoxaflor (400µM)	61.765 ± 2.230	103.32 ± 8.33
40		51.164 ± 3.229	85.49 ± 7.21
60		57.778 ± 3.978	96.52 ± 8.20
80		56.686 ± 4.609	94.69 ± 8.05
100		59.241 ± 5.437	98.96 ± 8.41
120		57.570 ± 2.060	96.17 ± 8.18
140	sulfoxaflor (2mM)	53.102 ± 2.516	88.70 ± 7.54
160		49.152 ± 6.907*	82.11 ± 6.98*
180		58.441 ± 3.285	97.62 ± 8.30
200		60.592 ± 2.302	101.22 ± 8.61
220		62.422 ± 4.101	104.27 ± 8.87
240		54.603 ± 3.138	91.21 ± 7.76
260	K^+ (50mM)	49.692 ± 5.211*	83.01 ± 7.06*
280		44.933 ± 4.164**	75.06 ± 6.38**

Data are means ± SEM. Means are the adjusted means from the mixed linear model with animal as the subject. SEM's are calculated from the residuals of the statistical model. % of baseline calculated from the adjusted means. P values for the comparison to baseline use Williams' test for sulfoxaflor and the multiple t-test for K^+. *p<0.05, **p<0.01 significantly different from baseline.

Histology

No observations / results reported. Measurements of analyte data are reported only from animals where probe membranes were correctly positioned (n=7). Rat 1 was excluded from the analysis as the probe location was found to be positioned slightly outside of the hypothalamus.

Conclusions

The aim of the project was to determine whether sulfoxaflor acting on central nicotinic receptors altered the efflux of dopamine from the neurones of the mediobasal hypothalamus, as a surrogate for dopamine in the median eminence, in conscious, freely moving rats when the compound was administered directly into this brain region by reverse dialysis. The experiments showed that sulfoxaflor produced concentration related increases in the extracellular level of dopamine in the mediobasal hypothalamus. The finding that a depolarising pulse of K^+ ions produced a transient and sharply delineated increase in dopamine efflux indicated that these hypothalamic dopaminergic neurones were viable and were normally responsive to physiological stimuli such as receptor mediated release of neurotransmitter. Relative to the magnitude of the effect of a depolarising pulse of K^+ ions, the increases evoked by sulfoxaflor were an average of 25% at the lower concentration and 43% at the higher concentration. In more absolute terms the 400µM concentration of sulfoxaflor elicited a 15% response above baseline, the 2mM concentration of sulfoxaflor elicited a 26% response above the initial baseline and 50mM K^+ elicited a 61% response above initial baseline responses (table 6.5.4.2-2). It is by no means certain that this response is linear or non-linear in nature, the baseline did not return to initial levels in between test article infusions. Nevertheless these actions were considered to be of pharmacological relevance and the findings support the hypothesis that sulfoxaflor increased dopaminergic neurotransmission in the mediobasal hypothalamus. In addition to measuring dopamine efflux in these microdialysis experiments, the extracellular concentrations of its two major metabolites, ie DOPAC and HVA, were also determined. Sulfoxaflor and K^+ ions increased the extracellular concentration of dopamine and produced a concomitant reduction in the concentration of HVA; but neither sulfoxaflor nor K^+ ions altered the extracellular concentration of DOPAC. The effects of a depolarising pulse of K^+ ions on the extracellular concentration of dopamine, DOPAC and HVA observed in these experiments were identical to those reported in a previous hypothalamic microdialysis study (*Matos et al., 1990*). Together, the current and previously published results indicate that sulfoxaflor increases dopamine secretion from hypothalamic dopaminergic neurones.

The increases in extracellular dopamine in the mediobasal hypothalamus were observed using concentrations of 400µM and 2.0mM sulfoxaflor in the dialysis fluid. However, they do not reflect the levels in the brain because the concentration of a compound or neurotransmitter on either side of the semi permeable membrane of the microdialysis probe does not reach full equilibrium. Experiments comparing the concentration of an analyte in a microdialysate with those present in a solution of known concentration generally reveal recovery rates of approximately 10% (though there is no data reported in this study as to what actual value is true). Thus, if a microdialysis probe is placed in a 1mM solution of a compound, the concentration of the compound measured in the dialysate will be assumed to be in the region of 0.1mM, i.e. 10-fold lower. This argument also applies in the reverse situation when compounds diffuse from the dialysis perfusion medium across the semi permeable membrane into the extracellular fluid. Thus, a concentration of 400µM of sulfoxaflor in the perfusion medium is assumed to equate to a concentration of approximately 40µM in the extracellular fluid. Because the rate of equilibrium is constant over a wide range of concentrations, the same correction factor also applies to the higher concentration of 2mM sulfoxaflor. On this basis, and because the blood-brain barrier surrounding the area of the hypothalamus is leaky (it is contained within the median eminence, one of the circumventricular organs and therefore one of the few sites in the brain that normally has an incomplete blood–brain barrier), it is reasonable to hypothesise that a circulating concentration of 40µM sulfoxaflor in the bloodstream would be capable of releasing dopamine from the TIDA neurones.

The dopaminergic neurones in the median eminence are unusual by virtue of having no terminal D2 autoreceptors to regulate neurotransmitter release (*Timmerman et al, 1995b*) and by having a very low capacity for dopamine reuptake (*Annunziato et al, 1980*). Both physiological characteristics are consistent with a neuro-secretory role for the TIDA dopaminergic neurones. Although the increases in dopamine efflux produced by sulfoxaflor appear to be relatively small in size compared for example with those induced by monoamine releasing agents, eg *d*-amphetamine or methamphetamine, it may be incorrect to assume that they are not of pharmacological relevance. *Kim and Han* (2009) compared the effects of nicotine and methamphetamine on extracellular dopamine concentrations in the striatum. In these experiments, the impact of the increases in synaptic dopamine concentrations on postsynaptic signal transduction were assessed by the displacement of [^{3}H]-raclopride (a D2 dopamine receptor antagonist) from D2 receptors. The results showed that although the maximum increase in dopamine efflux evoked by nicotine (approximately 150%) was small in comparison to that produced by methamphetamine (approximately 1500%) both drugs produced an approximately 40% displacement of [^{3}H]-raclopride binding possibly showing that their actions to enhance dopaminergic signalling were approximately equivalent. Taking these findings together with the reported lack of auto inhibitory control of TIDA dopaminergic neurones, it is possible that the increases in dopamine efflux evoked by sulfoxaflor would be of pharmacological and physiological relevance. This is presumably the case in the context of chronic stimulation such as long term exposure to sulfoxaflor as occurs in the rat combined chronic toxicity and carcinogenicity study.

Only the effects of acute administration of sulfoxaflor have been determined in the current study; however, the effects of repeated administration of nicotine on dopamine efflux have been investigated by other researchers (*Marshall et al, 1997*). Repeated subcutaneous administration of nicotine to rats for 7 days when given either by injection or by continuous infusion using osmotic mini pumps resulted in a marked potentiation of the action of reverse dialysed nicotine to increase dopamine efflux in both the striatum and nucleus accumbens. Translating these findings to sulfoxaflor predicts that there would be no tachyphylaxis to its effects on dopamine efflux from TIDA neurones when given repeatedly, and furthermore, the magnitude of its effect may increase when the compound is administered for prolonged periods.

In summary, sulfoxaflor caused concentration related increases in local dopamine concentrations possibly because of increased release or enhancement of synaptic longevity due to slower synaptic reuptake or both, from the mediobasal hypothalamic dopaminergic neurones when reverse dialysed into this brain region. The effects are potentially of pharmacological and physiological relevance. The data support the hypothesis that sulfoxaflor may increase dopamine efflux from TIDA neurones in the median eminence and that this effect would be predicted to result in a decrease of prolactin secretion by the anterior pituitary gland.

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Study 9: MoA Study: Screening for Oestrogen Receptor and Androgen Receptor Binding and Transactivation and Aromatase Inhibition. DAR Section B.6.5.4.3.

A series of *in vitro* screening tests with sulfoxaflor to evaluate for oestrogen receptor (ERα) alpha and androgen receptor (AR) binding, ER and AR transactivation (agonism and antagonism), and aromatase inhibition, did not indicate changes consistent with endocrine-mediated alterations. Androgen receptor antagonists compete with testosterone and dihydrotestosterone for binding to the androgen receptor. This competition can reduce the androgenic signal to the hypothalamus and adenohypophysis, resulting in an increase in LH secretion with a concomitant elevation of testosterone secretion, resulting in the development of LCTs. Results obtained with sulfoxaflor (section B.6.5.4.3; Toole, 2011), showed that it may have bound non-specifically to a fragment (i.e., ligand binding domain) of the AR in a non-cell-based binding assay but because there was no effect on agonism or antagonism within the AR transactivation assay there may be no biological relevance of this potential AR binding result. Sulfoxaflor was identified as a non-binder in the oestrogen receptor α (ERα) Fluorescence Polarization (FP) assay because no displacement of the fluormone from the oestrogen receptor occurred. The ER transactivation assay was also negative for agonism or antagonism by sulfoxaflor. Concurrent positive controls indicated specificity of the system to identify an ER-binding compound. In two independent runs of the aromatase assay sulfoxaflor did not inhibit CYP19. Concurrent positive controls indicated specificity of the system to identify an aromatase inhibitor.

Report: Toole, C. (2011). XDE-208 Technical: Screening for Estrogen Receptor and Androgen Receptor Binding and Transactivation and Aromatase Inhibition. CeeTox, Inc. 4717 Campus Drive, Kalamazoo, Michigan, USA. Unpublished.

Report No.: DR-0404-3134-123; Report Number: 9115-100297.

Dates: 2011

Guidelines: Non-guideline.

GLP: No. Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were not provided.

Deviations: This is an acceptable though non-guideline study, it is considered supplementary to the long-term chronic / carcinogenicity studies and suitable for a MoA investigation.

Deficiencies: No.

Executive Summary: These studies describe the ability of sulfoxaflor to interact with the oestrogen and androgen receptors and inhibit aromatase activity. Sulfoxaflor is identified as a non-binder in the oestrogen receptor alpha (ERα) fluorescence polarisation (FP) assay as no displacement of the fluormone from the oestrogen receptor occurred. Sulfoxaflor is

categorised as a potential binder in the androgen receptor (AR) FP assay as the binding curve exceeded the required 50% displacement of the fluormone from the receptor. The AR and ER transactivation assays were negative for agonism or antagonism by sulfoxaflor. Based on this, sulfoxaflor-related non-specific interaction cannot be ruled out as a potential mechanism of action for the response observed in the AR-binding assay, as no biological effect was identified in an AR-mediated transactivation assay. The aromatase assay determined that sulfoxaflor did not inhibit aromatase (CYP19) activity. The results from the five different *in vitro* screening tests with sulfoxaflor described further did not indicate changes consistent with endocrine-mediated alterations.

Sulfoxaflor was assessed in 5 different assays in order to determine its potential for endocrine activity. The assays performed were as follows: ERα binding (FP), AR binding (FP), ER and AR transactivation (agonism and antagonism), and AR aromatase inhibition. The top concentration of sulfoxaflor for use in the assays was 10^{-3}M. Two independent runs of each assay were performed. Sulfoxaflor did not demonstrate any agonism or antagonism in the ER and AR transactivation assays. Reference controls demonstrated that the systems were performing as expected and able to detect mild agonism and antagonism for both ER and AR.

Materials and Methods

Materials:

1	**Test Material:**	Sulfoxaflor
	Synonyms:	XDE-208; (N-(Methyloxido(1-(6-(trifluoromethyl)-3-pyridinyl)ethyl)-λ^4-sulfanylidene)-cyanamide); [1-(6-Trifluoromethylpyridin-3-yl)ethyl](methyl)-oxido-λ^4-sulfanylidenecyanamide; Sulfoximine; X11422208; XR-208.
	Description:	White Solid
	Lot/Batch #:	Lot # E2162-34, TSN003725-001.
	Purity:	96.6% (w/w); as two diastereomers in 48.4 / 47.4% ratio
	CAS #:	946578-00-3

Study Design:

Vehicle: Dimethyl sulfoxide (DMSO) was selected as a suitable vehicle for sulfoxaflor for all assays, therefore, solutions with a sulfoxaflor concentration of up to 10^{-3}M (the limit concentration for the assay) was prepared whilst limiting the final concentration of DMSO.

Cell lines: T47D-KBluc Cell Line: The stably transfected hERα-T47D-KBluc cell line was used in this study to evaluate ER agonism and antagonism by the test substance sulfoxaflor. This cell line was obtained from ATCC (CRL-2865). The T47D-KBluc cell line was certified to be free of mycoplasma as tested and verified by ATCC. T47D-KBluc was derived from the parental cell line, T47D, transfected with pGL2.TATA.Inr.luc.neo which contains 3 oestrogen response elements (EREs) upstream of a *luc* reporter gene. This cell model is used as a sensitive screen for chemicals to evaluate oestrogenic (agonism) or anti-oestrogenic (antagonism) activity using a simple luciferase readout. The cells used in this study had been

in culture prior to seeding into plates on passages 59 and 63 for use in the ER transactivation assay.

The cells were grown in a Forma Scientific CO_2 water jacketed Model 3110 incubator equipped with HEPA filter system and set at 37°C with CO_2 at 5%. Prior to the addition of the test compounds the growth media (RPMI 1640 medium, Sigma R8755 with 10% serum, 500mg/L glucose, 10mM HEPES, 100mg/L sodium pyruvate and 1.5g/L sodium bicarbonate), containing antibiotics was removed and the cells were maintained for 5 to 10 days in assay medium containing 10% Dextran/charcoal treated fetal bovine serum (Gibco 16140 treated in house). Approximate doubling time for T47D-KBluc cells was 32-36 hours. Cells were seeded into individual wells of a 96 well culture plate(s) at approximately 10,000 cells/well at passages 59 and 63.

MDA-kb2 Cell Line: The stably transfected hAR-MDA-kb2 cell line was used in this study to evaluate AR agonism and antagonism by the test substance sulfoxaflor. This cell line was obtained from ATCC (CRL-2713). The MDA-kb2 cell line was certified to be free of mycoplasma as tested and verified by ATCC. The MDA-kb2 cell line was derived from a human breast carcinoma cells, MDA-MB-453, by stable transfection with a mouse mammary tumour virus (MMTV) luciferase-neo reporter gene construct. This assay using an androgen receptor is a valuable tool for screening for potential androgen agonists and antagonists. The cells used in this study had been in culture prior to seeding into 96 well plates on passage 45 for use in the AR transactivation assays.

The cells were grown in a Forma Scientific CO_2 water jacketed Model 3110 incubator equipped with HEPA filter system and set at 37°C without CO_2. Cells were initially grown in Leibovitz's L-15 medium (Hyclone SH30525.02) containing 10% Fetal Bovine Serum (FBS, Gibco 16000) without antibiotics at 37°C and without CO_2. The doubling time for these cells is approximately 40 - 48 hr. Cells were seeded into individual wells of a 96 well culture plate at passage 45 at approximately 10,000 cells/well.

Chemical Exposure and Assay Plate Organization for Transactivation Assays: MDA-kb2 and T47D-KBluc cells were grown for an additional 20 - 24 hours prior to the addition of test and reference compounds. For both AR and ER transactivation assays, the reference chemicals and the test substance were dissolved in DMSO to make a 2× stock solution of each desired concentration. When added to the cell culture plates this would yield the final serial concentrations for the reference chemicals and for the test substance sulfoxaflor (concentrations of -6.52, -6.0, -5.52, -5.0, -4.52, -4.0, -3.52, and -3.0 logM), DMSO held constant at 0.5% (v/v). Test chemicals were added to the cells after the culture medium was aspirated from the cells. 50 µL of the 2× concentrated stock solutions were added to 96 well plates containing 50 µL/well assay media (with DMSO, agonist, or antagonist) for a final volume of 100µL/well. Assay plates for agonism assays were organised as detailed below. After adding the reference chemicals/test substance, the plates were incubated for approximately 24 hours.

	1	2	3	4	5	6	7	8	9	10	11	12

A	Blank*	*** (nM)	VC**	VC	Conc. 1	Conc. 2	Conc. 3	Conc. 4	Conc. 5	Conc. 6	Conc. 7	Conc. 8
B	↓***	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
C	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
D	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
E	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
F	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
G	----------As above + antagonist (1 μM ICI 182,780 (ER) or 1 μM Nilutamide (AR))----------											
H	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓

*Blank wells contain media only (no cells); **Vehicle control (VC) wells contain cells and media + 0.5% (v/v) DMSO; ***1 nM 17β-estradiol (E2) or 10 nM dihydrotestosterone (DHT) Maximal induction control wells; ****↓ Indicates the composition of the well is identical to the well directly above it

In view of the short-term nature of studies of this type, no analyses of stability, homogeneity or achieved concentration(s) were carried out on preparations of the test substance or positive control chemicals. For the positive control compounds, stability was demonstrated by an appropriate response in the assay system (as compared to historical data).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank*	*** (nM)	VC**	VC	Conc. 1	Conc. 2	Conc. 3	Conc. 4	Conc. 5	Conc. 6	Conc. 7	Conc. 8
B	↓***	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
C	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
D	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
E	----------As above + antagonist (100 nM E2 (ER) or 1000 nM DHT (AR))----------											
F	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
G	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
H	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓

*Blank wells contain media only (no cells); **Vehicle control (VC) wells contain cells and media + 0.5% (v/v) DMSO; ***1nM 17β-estradiol (E2) or 10 nM dihydrotestosterone (DHT) Maximal induction control wells; ****↓ Indicates the composition of the well is identical to the well directly above it. Rows A-D are low levels of antagonist (0.1 nM E2 for ER or 1 nM DHT). Rows E-H are high levels of antagonist (100 nM E2 for ER or 1000 nM DHT for AR).

For antagonism assays, MDA-kb2 and T47D-KBluc cells were seeded into individual 96 well culture plates at a density of ~10,000 cells/well in the media and grown for an additional 20 – 24 hours prior to the addition of test and reference compounds. Assay plates for antagonism assays were organised as detailed below:

Assay types and Methods:

(1) Cytotoxicity Assay: Cell viability was monitored by propidium iodide (PI) uptake. PI is a dye that cannot cross the plasma membrane of intact and viable healthy cells. Cells that were dead or dying have weakened plasma membrane which allowed PI to enter the cytosol of the damaged cells. Once inside the cell, PI intercalated into DNA/RNA and yielded a fluorescent signal. The intensity of the fluorescent signal was directly proportional to cell viability. As PI is a light sensitive compound all procedures were conducted under low light conditions.

Cells were seeded on a black-walled 96 well cell culture plate. Following chemical exposure, the growth medium was removed and 50µL of a PI working solution (4.0µM in phosphate buffered saline) was added to each well. Background fluorescence was evaluated by measuring fluorescence on a Packard Fusion fluorescence plate reader at an excitation wavelength of 544nm and an emission wavelength of 612nm. Following this determination, 50µL of a 2% (v/v) triton X-100 solution was added to each well and the plate was incubated at room temperature for 15 ± 5 minutes before measuring fluorescence at the same wavelengths. The background-corrected fluorescence was calculated for each well by subtracting the results of the first read from the results of the second read. The change in cell viability was determined by comparing treated wells to the vehicle control wells. ≥A20% reduction in cell viability was considered evidence of cytotoxicity.

(2) Precipitation Assay: Precipitation was determined by a light scattering procedure that uses nephelometry (Nepheloskan Ascent by Labsystems). If a concentration consistently gave a signal >3 times the vehicle control signal, that concentration was considered to have precipitation.

(3) Transcriptional Activation (Luciferase) Assay: A luciferase assay was performed as described in CeeTox Standard Operating Protocol (SOP) 2041 using the reagents listed below.

Reagent	Supplier	Catalog #
Trisma Base	Sigma	T6066
Magnesium Chloride	Sigma	M2393
	EMD	Mx0045-2
EDTA	Sigma	E5134
Dithiothreitol	Sigma	D9779
		D6032
ATP	Sigma	A2383
Coenzyme A	Sigma	C3019
AMP	Sigma	A1752
Luciferin	Promega	E160E

Glycerol	Sigma	G5516
Triton-X100	Sigma	T8787
Bovine Serum Albumin	Sigma	A9418
CDTA	Sigma	D0922

(4) Fluorescence Polarisation Assays: Stock solutions of the test materials were prepared in DMSO on the day of testing. All reagents and solutions were used at ambient laboratory temperature. Dihydrotestosterone (DHT) was used as the positive control for AR binding. 17β oestradiol was used as the positive control for the ERα binding. Reference control materials Methoxychlor and Nonylphenol were also evaluated and compared to historical data. Vehicle controls (DMSO) were also included as negative controls on each plate.

Briefly, the primary stock solution for the test compound sulfoxaflor was prepared in DMSO along with reference controls. The final reaction mixture was prepared by adding the appropriate amount of the test compound stock solution directly into white-sided 96-well assay plates containing the AR/ER assay buffer. The solutions were mixed by repipetting a minimum of six times. This process provided the 2× target test concentrations with a 2× DMSO concentration of 1%. Compound precipitation was evaluated at this point using a Nepheloskan (Thermo-LabSystems) system.

(4a) AR binding Assay: A 2nM solution of the Fluormone™ AR Green was prepared according to the manufacturer's instructions. An aliquot of each of the diluted test material (20µl) was removed from the 96-well white assay plate and used for diluting the test material and determining precipitation by placing into a black 384 well microplate in triplicate. Additionally 3 or more wells were filled with 20µl of AR Green assay buffer with 1% DMSO and Fluormone to provide a "free-ligand" control. Another 20µl of the assay buffer containing 20µM of the standard ligand control was added to 3 or more additional wells to serve as "total displacement" (Nonspecific binding, NSB) controls upon addition of Fluormone. The plates were tapped gently to remove air bubbles or centrifuged at 750×g for 30 seconds.

At this point the compounds were evaluated for fluorescent properties that might interfere with the fluorescence polarisation assay. The compounds in assay buffer was read with non-polarised light at the same excitation and emission wavelengths used in the fluorescence polarisation assay with a PerkinElmer EnVision 2100 Multilabel spectrophotometer. Values that were 20% above background controls, if present, were eliminated from the analysis. The assay was continued by adding 20µL of 2nM Fluormone + 50nM Androgen receptor (Androgen receptor ligand binding domain, AR-LBD) to the wells containing 20µL of the diluted test compounds, but not to the wells designated as "free-ligand" controls. The free-ligand control wells received 20µL of AR Green assay buffer without receptor. Final concentrations in the test wells were 1nM Fluormone™ AL Green and 25nM AR-LBD (1% DMSO). The plate was tapped gently and centrifuged as above to remove air bubbles. The AR binding plates were incubated for 4-8 hr in the dark at ambient temperature. Following the incubation period the assay plate was read with a PerkinElmer Envision spectrophotometer at excitation and emission wave lengths of 480nm and 535nm respectively.

(4b) ER binding Assay: A 2nM solution of the Fluormone ES2 buffer was prepared according to the manufacturer's instructions. An aliquot of each of the diluted test material (30µl) was removed from the 96-well white assay plate used for diluting the test material and determining precipitation, and placed into a black 384 well Matrical microplate in triplicate. Additionally 3 wells were filled with 30µl of ER buffer with 1% DMSO to provide a "free-ligand" control upon addition of Fluormone. Another 30µl of the ER buffer containing 20µM of the standard ligand control was added to 3 or more additional wells to serve as "total displacement" controls upon addition of Fluormone. The plates were tapped gently to remove air bubbles and centrifuged at 750×g for approximately 30 seconds.

At this point the compounds were evaluated for fluorescent properties that might interfere with the fluorescence polarisation assay. The compounds in assay buffer was read with non-polarised light at the same excitation and emission wavelengths used in the fluorescence polarisation assay with a PerkinElmer EnVision 2100 Multilabel spectrophotometer. Values that were 20% above background controls, if present, were eliminated from the analysis.

The assay was continued by adding 30µL of 2nM Fluormone + 15nM oestrogen receptor alpha to the wells containing 30µL of the diluted test compounds, but not to the wells designated as "free-ligand" controls. The free-ligand control wells received 30µL of Fluormone ES2 buffer without oestrogen receptor alpha. Final concentrations in the test wells were 1nM Fluormone and 7.5nM oestrogen receptor alpha. The plate was tapped gently and centrifuged as above to remove air bubbles. The ER binding plates were then incubated in the dark for 2 - 4 hours at ambient temperature. Following the incubation period the assay plate was read with a PerkinElmer Envision spectrophotometer at excitation and emission wave lengths of 480nm and 535nm respectively.

(5) Aromatase Assay: The amount of oestrone formed was determined by dividing the total amount of $^{3}H_2O$ formed by the specific activity of the substrate [^{3}H]-androstenediene (expressed in DPM/nmol). The activity of the enzyme reaction was expressed in nmol/mg-protein/min and was calculated by dividing the amount of oestrone formed by the product of mg microsomal protein used times the incubation time, i.e., 15 minutes. Three types of control samples were included in each run which include:

- Full enzyme (aromatase) activity controls (substrate, NADPH, propylene glycol, buffer, vehicle (used for preparation of test substance solutions) and microsomes).

- Background activity controls (all components that are in the full aromatase activity controls except NADPH).

- Positive inhibitor controls (4-OH-androstenedione also known as ASDN, run at eight concentrations in the same manner as test substances).

Four test tubes of the full enzyme activity control and background activity controls were included with each run. The full enzyme and background activity controls sets were split so that two tubes (of each control type) were run at the beginning and two at the end of each run. The positive control was tested at eight concentrations in each run. All controls were treated the same as the other samples. The radiochemical content using liquid scintillation spectrometry (LSS). Radiolabel found in the aqueous fractions represents $^{3}H_2O$ formed.

Relevant data was entered into the assay spreadsheet for calculations of aromatase activity and percent control. The spreadsheet calculates the DPM/mL for each aliquot of the extracted

aqueous incubation mixture and average DPM/mL and total DPM for each aqueous portion (after extraction). The volume (mL) of substrate solution added to the incubation multiplied by the substrates specific activity (DPM/mL) yields the total DPM present in the assay tube at initiation. The total DPM remaining in the aqueous portion after extraction divided by the total DPM present in the assay tube at initiation times 100 yields the percent of the substrate that was converted to product. The DPM is then converted to nmol product formed by dividing by the substrate specific activity (DPM/nmol). The activity of the enzyme reaction is expressed in nmol (mg product)-1min-1 and average activity in the full activity control samples was calculated. Percent of control activity remaining in the presence of the various inhibitor concentrations, including the positive control, was calculated.

Data from this assay was used to classify sulfoxaflor according to its ability to inhibit aromatase (CYP19). To be classed as an inhibitor, the data must fit the 4-parameter regression model to yield an inhibition curve and result in greater than 50% inhibition at the highest concentration.

Transcriptional activation assay (agonism) data analysis and interpretation:

To determine the relative transcriptional activity (agonism) as compared to the positive control (PC) (1nM 17β-oestradiol), the luminescence data from each plate were measured using a luminescence counter. The data from the counter were transferred to spreadsheets to determine standard statistical parameters such as the Mean, Standard Deviation (SD), Standard Error (SE) of the Mean, and percent (%) Coefficient of Variation (CV). After determining the mean response, the mean values of response were reviewed for outlier values. The outlier values were calculated as the mean ± 2 SD. Any values outside of these parameters were eliminated from the final analysis. All processed data was examined to determine if negative and positive induction controls within each plate were also within acceptable limits. *The acceptance criteria used were:*

- vehicle is less than 10 fold over background, **and** the ratio of positive control to VC was greater than 3-fold.

Each data point was normalised to the average of the vehicle-only treated control (fold induction). The final Fold Induction results were then transferred as individual data points in plate block format.

Test substance was considered negative if:

- the maximum response relative to the positive control (% Maximal Induction) was < 20% for hER/AR agonism.
- The test substance was considered negative if % maximal induction was < 20% in at least 2 definitive runs of the transcriptional activation assay.

Test substance was considered positive if:

- The % maximal induction was ≥ 20% in at least 2 definitive runs of the transcriptional activation assay or

- For antagonism, the % of maximal induction needs to demonstrate a dose response incorporating at least 2 points and fall below 50% to be considered positive **and** the differential (antagonist % of maximal control for between the high antagonist control

wells (mean) minus the antagonist % of maximal control for low antagonist) must be 50% or greater.

Fluorescence polarisation assay data analysis and interpretation:

The PerkinElmer Wallac EnVision Manager v 1.07 software calculated the mP (milli-polarization) value of the Fluormone bound with receptor. mP was calculated using the formula mP = 1000 * (S – G * P) / (S + G * P) where S = detector 2 (same plane), P = detector 1 (perpendicular plane), and G (grating) = 1. The raw data and mP values were transferred into Microsoft Excel worksheets for labelling and calculation of means and standard deviations of control samples. The compound numbers were added to the right of the data. The data was then transferred into GraphPad Prism (or Activity Base) for plotting and regression curve analysis. Test concentrations were transformed into Log (x) values and represented the X-axis of the binding graphs. The response values were normalized to 100% response (receptor plus Fluormone ligand without competitor) and 0% response (Fluormone in assay vehicle with an excess of standard ligand to ensure maximal displacement and typical non specific binding).

Outlier values were excluded from the Prism5 data tables. A value was excluded if it was markedly inconsistent from other values on same plate. The rationale was to exclude no more than one value of triplicates if possible. The "100% bound" controls had a total of 6 replicates: 2 sets of 3 control wells, therefore outlier selection was made by visually evaluating all 6 replicates in the data table. Only the first non-excluded row from the top of the table was used by Prism5 to normalize the data. If the first row was excluded entirely, Prism5 uses the mean of the second row as the 100% value. Each GraphPad Prism chemical file has at least two data tables. The first was used to plot the data means for each concentration. The second data table created for each compound was used for the regression analysis curve. Therefore, concentrations that exceeded the precipitation limits or caused signal interference were excluded from the regression analysis but still included on the plots.

Ligand displacement curves were analyzed by performing regression analysis using the equation for one site competition (Y = bottom + (top-bottom) / 1 + 10 ^ (X-logEC_{50})) with a curve bottom analysis constraint set to 0% mP. Note that the graphical data depicted individual data points with the regression curve. In cases where insolubility occurred at concentrations before 50% displacement, the regression analysis was not plotted. The indicators marking the compound concentrations where precipitation was observed at a consistent signal ≥ 3 times the vehicle control values were manually added to the graphs.

Evaluation Guidelines for Endocrine Receptor Binding Data:

If:	Interpretation:
binding curve crosses 50%	Potential binder
crosses 75%, but not 50%	Equivocal
data does not fit model or curve does not cross 75%	non-binders

Adopted from TECHNICAL REVIEW DOCUMENT for ENCOCRINE DISTRUPTER SCREENING PROGRAM (EDSP) PROPOSED TIER 1 SCREENING BATTERY. U.S. Environmental Protection Agency Office of Prevention, Pesticides, and Toxic Substances Office of Science Coordination and Policy

Results and Discussion

Transcriptional ER and AR Activation Assay.

Two independent runs of the assay were conducted for the mean luciferase activity. The PC (1 nM 17β-oestradiol). was greater than 4-fold that of the mean luciferase activity of the VC on each plate and the mean of the VC wells divided by the average background wells was less than 20. For each transactivation assay the reference compounds, 17β-oestradiol (strong agonist), methoxychlor (mild agonist), and ICI 182,780 (strong antagonist) for ER; and the reference compounds DHT (strong agonist), nilutamide (strong antagonist), and nonylphenol (mild antagonist) for AR, were within historical limits of the data. There was no data for sulfoxaflor excluded from either evaluation or interpretation due to excessive cytotoxicity (< 80% viable) or precipitation (>3 NRU) in any independent run of the assay. Agonism and Antagonism plates both passed performance criteria.

In two independent runs of the assay, sulfoxaflor did not result in an increase in luciferase activity at any of the concentrations tested for either AR or ER agonism nor were there any indications of antagonism.

1. For sulfoxaflor, the maximum response relative to the positive control (% maximal induction) was determined to be less than 20% for each of the two receptors (two runs for AR agonism and three runs for ER agonism) and therefore considered negative for agonism (figure 6.5.4.3-1, AR transactivation and figure 6.5.4.3-2, ERα transactivation).

2. Sulfoxaflor was negative as the % maximal induction was < 20% in the 2 definitive runs of the transcriptional activation assay for AR and 3 definitive runs for ERα.

3. For antagonism, the % of maximal induction of sulfoxaflor did not demonstrate a dose response incorporating at least 2 points nor did it fall below 50%.

4. The differential (antagonist % of Maximal Control for between the high antagonist control wells (mean) minus the antagonist % of Maximal Control for low antagonist) must be 50% or greater to be considered significant. Sulfoxaflor was no different to control.

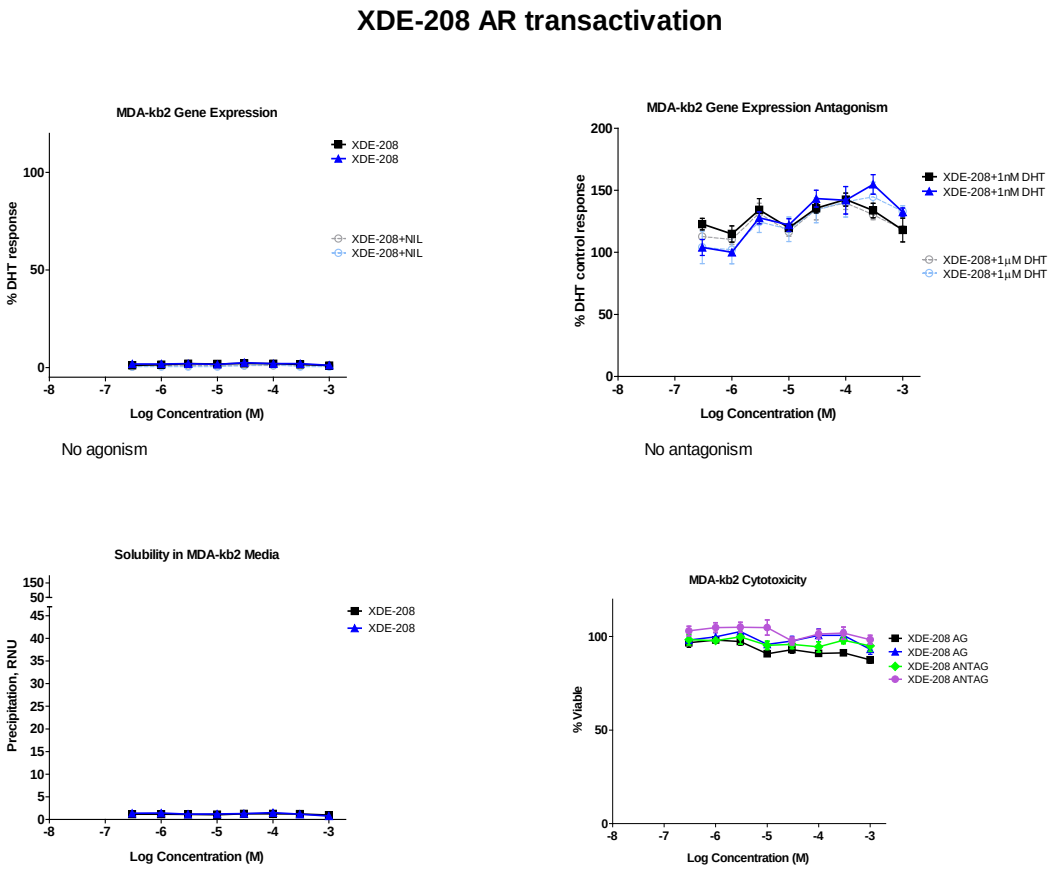


Table 4.10.3.1.Study 9.1 (DAR Figure 6.5.4.3-1): Sulfoxaflor – AR Transcriptional Activation (agonism and antagonism).

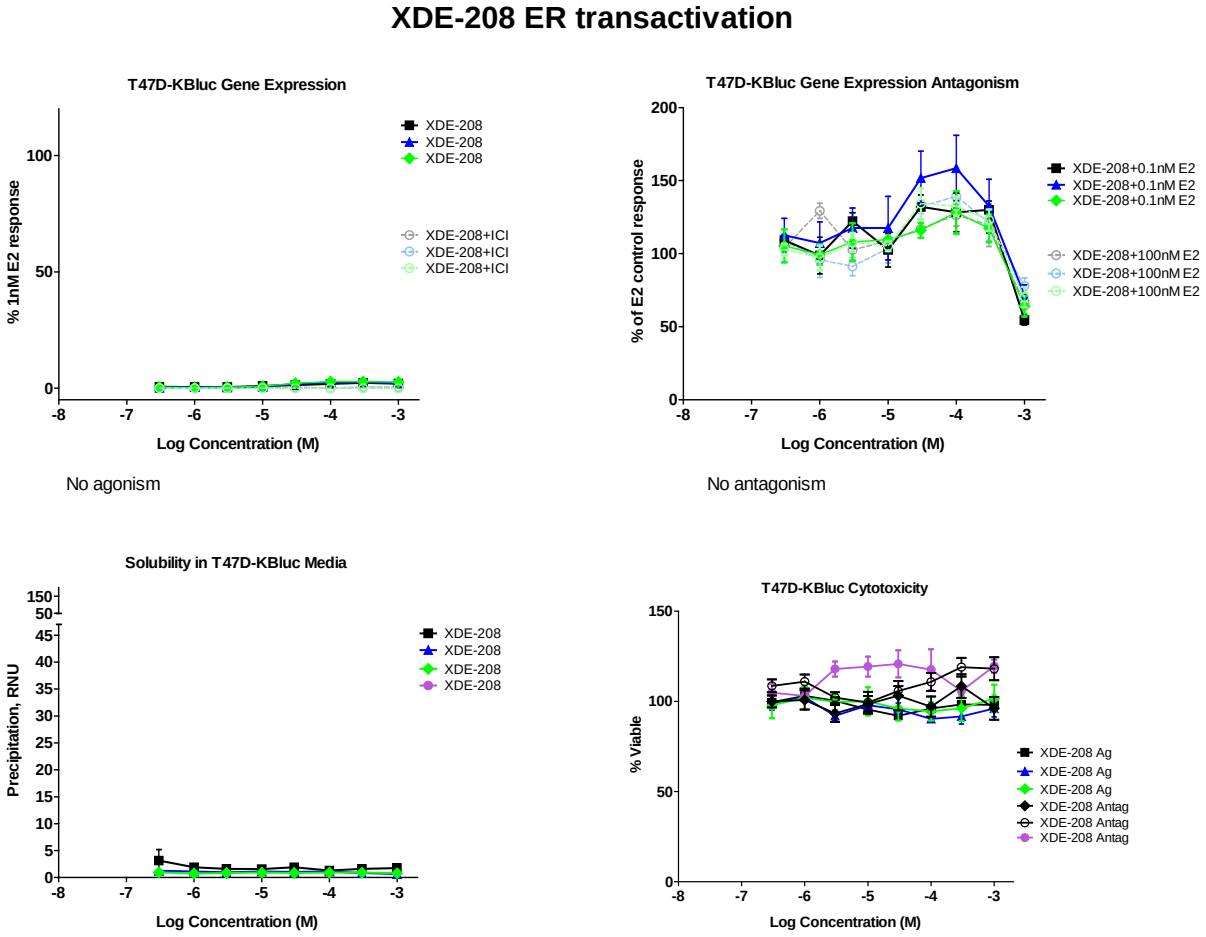


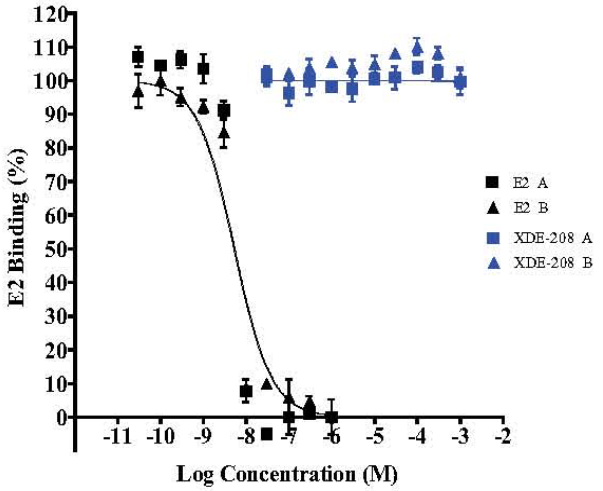
Table 4.10.3.1.Study 9.2 (DAR Figure 6.5.4.3-2): Sulfoxaflor – ER Transcriptional Activation (agonism and antagonism).

Sulfoxaflor was determined to be negative for agonism and antagonism in the AR and ER transactivation assays. No values were eliminated from the analysis due to cytotoxicity or precipitation.

ER and AR Binding Assay.

The oestrogen receptor (ER) and androgen receptor (AR) binding potential of sulfoxaflor was assessed in separate fluorescent binding polarisation assays through the displacement of a fluorescent ligand, Fluormone. The ability of sulfoxaflor to act as a potential binder for human oestrogen receptor alpha (hERα) and AR was assessed using the full-length ERα and the AR ligand binding domain, respectively. Sulfoxaflor was identified as a non-binder in the hERα Fluorescence Polarisation (FP) assay as no displacement of the fluormone from the oestrogen receptor occurred – the binding curve did not cross 75% for ERα (figure 6.5.4.3-3a). The AR binding results indicated that sulfoxaflor could be a potential binder of the androgen receptor – the binding curve for both assay runs crossed 50% (figure 6.5.4.3-3b).

Table 4.10.3.1.Study 9.3 (DAR Figure 6.5.4.3-3a): ERα FP Binding Assay

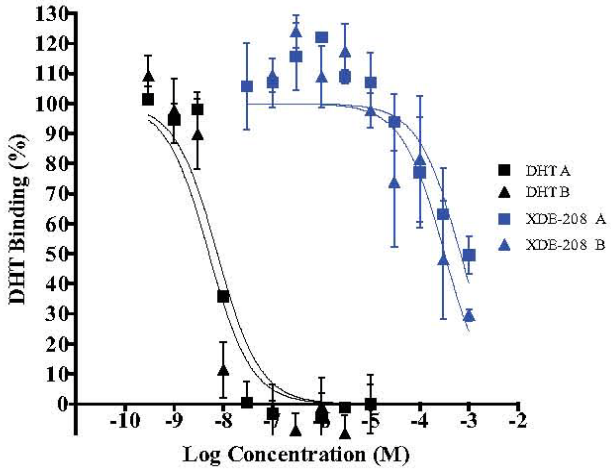


	E2 IC50 (M)
A	5.47e-009
B	5.14e-009

	Hill Slope
E2 A	-4.04
E2 B	-3.21

	Solubility limit (log M)
XDE-208 A	0.00
XDE-208 B	0.00

Table 4.10.3.1.Study 9.4 (DAR Figure 6.5.4.3-3b): AR FP Binding Assay



	DHT IC50 (M)	XDE-208 IC50 (M)
A	7.63e-009	6.72e-004
B	5.12e-009	3.19e-004

	Hill Slope
DHT A	-3.85
DHT B	-3.59
XDE-208 A	-0.82
XDE-208 B	-0.89

	Solubility limit (log M)
XDE-208 A	0.00
XDE-208 B	0.00

The ERα is a full length receptor and represents a standard *in vitro* approach for a receptor binding assay, while the AR used in these assays consist of only the ligand binding domain (LBD) which is a truncated version of the full length receptor. In neither of these binding assays are the cofactors present as in the *in vivo* situation. Classically, interaction of native steroid hormones with their cytosolic/nuclear receptors results in conformational changes, receptor dimerisation, binding of the complex to specific DNA sequences (response elements) leading to recruitment of coactivator proteins. These complexes can then initiate or inhibit the transcription of hormonally regulated genes and subsequent cellular functions. The lack of a response in the transactivation assay for sulfoxaflor AR agonism where the cofactors and complete system of activation is present would tend to be more favored over the AR binding fluorescence polarisation results.

In two independent runs of the AR binding assay, sulfoxaflor exhibited a binding curve that passed 50% which categorises this test substance as a potential binder. The relative binding affinity of sulfoxaflor (table 6.5.4.3-1 and table 6.5.4.3-2) as compared to DHT (6.37×10^{-9}) was weak with a mean value of approximately 5×10^{-4}. Furthermore, the AR transactivation assays do not support the binding data identified in the FP binding assay.

Table 4.10.3.1.Study 9.5 (DAR Table 6.5.4.3-1): AR FP binding model data summary

	DHT A	sulfoxaflor A	DHT B	sulfoxaflor B
One-site competition Best Fit Values				
Bottom	0.0	0.0	0.0	0.0
Top	100.0	100.0	100.0	100.0
LogEC50	-8.118	-3.172	-8.291	-3.496
EC50	**7.628e-009**	**6.772e-004**	**5.121e-009**	**3.193e-004**
Std Error				
LogEC50	0.1002	0.1414	0.1384	0.1683
Goodness of Fit				
Degrees of Freedom	29	29	29	29
R^2	0.8965	0.5209	0.8243	0.5652
Abs Sum of Squares	6683	10901	12714	17797
Sy.x Constraints	15.18	19.39	20.94	24.77
Bottom	0.0	0.0	0.0	0.0
Top	100.0	100.0	100.0	100.0
No. Points Analyzed	30	30	30	30

Table 4.10.3.1.Study 9.6 (DAR Table 6.5.4.3-2): Relative binding affinity of sulfoxaflor in AR FP assay (mean of run A and B)

AR Binding	IC_{50} Mean (M)	IC_{50} Std Error	RBA Mean
DHT	6.37×10^{-9}	1.25×10^{-9}	NA
Sulfoxaflor	4.96×10^{-4}	1.76×10^{-4}	1.37×10^{-3}

In two independent runs of the ERα binding assay, sulfoxaflor did not displace the fluormone from the full length oestrogen receptor and no binding occurred (table 6.5.4.3-3 and table 6.5.4.3-4). The relative binding affinity of sulfoxaflor (table 6.5.4.3-3) as compared to E2 (5.31×10^{-9}) was virtually non-existent with a mean value of approximately 0.12. The ER transactivation assay supported the binding data and sulfoxaflor is not determined to interfere with oestrogen receptor binding. Concurrent positive controls indicated specificity of the system to identify an ER binding compound.

Table 4.10.3.1.Study 9.7 (DAR Table 6.5.4.3-3): ERα binding model data summary

	E2 A	sulfoxaflor A	E2 B	sulfoxaflor B
One-site competition Best Fit Values				
Bottom	0.0	0.0	0.0	0.0
Top	100.0	100.0	100.0	100.0
LogEC50	-8.262	-0.8599	-8.289	-0.1256
EC50	**5.473e-009**	**0.1381**	**5.142e-009**	**1.335**
95% Confidence Interv.				
LogEC50	-8.488 to -8.035	-4.108 to 2.388	-8.453 to -8.125	-29.28 to 59.54

EC50	3.249e-009 to 9.221e-009	7.806e-005 to 244.2	3.524e-009 to 7.503e-009	5.194e-030 to 3.432e+029
Goodness of Fit				
Degrees of Freedom	29	29	29	29
R^2	0.8967	-0.04236	0.9278	-0.1274
Abs Sum of Squares	8159	654.8	4283	588.2
Sy.x Constraints	16.77	4.752	12.15	4.504
Bottom	0.0	0.0	0.0	0.0
Top	100.0	100.0	100.0	100.0
No. Points Analyzed	30	30	30	30

Aromatase Assay.

In a screening assay for androgen receptor (AR) aromatase inhibition sulfoxaflor was evaluated by measuring the conversion of an androgen to an oestrogen using recombinant microsomes. Specifically, radioactive substrate (^{3}H-androstenediene) and NADPH were added to microsomes containing aromatase (CYP19) and reductase complex. ^3H_2O released during the conversion of androstenediene to oestrogen was quantitated as a direct measurement of aromatase activity per unit reaction time. Competitive inhibition of aromatase activity by sulfoxaflor was then evaluated. Aromatase inhibition assays were also run in duplicate. 4-OH-androstenedione (ASDN) was used as the positive inhibitor reference control (it is a well characterised inhibitor of aromatase).

Full enzyme activity controls (FEAC) and background activity controls (BAC) when averaged and expressed as percent of control were approximately 100 and 0 % respectively. The reference control ASDN was used as the positive control for each independent run of the assay. Hill Slopes, IC_{50}, Top% and Bottom % were evaluated against the criteria outlined in Endocrine Disruptor Screening Program Guidelines OPPTS 890.1200 Aromatase (Human Recombinant).

Sulfoxaflor was determined to be negative for aromatase inhibition in this assay. The combined curves (run 1 and run 2) closely resemble each other despite the slight differences in the reference control ASDN. Figure 6.5.4.3-4 shows both curves for ASDN (positive reference control) and sulfoxaflor. Each run pair closely resembles each other (sulfoxaflor run 1 is very similar to sulfoxaflor run 2 and ASDN run 1 is similar to run 2).

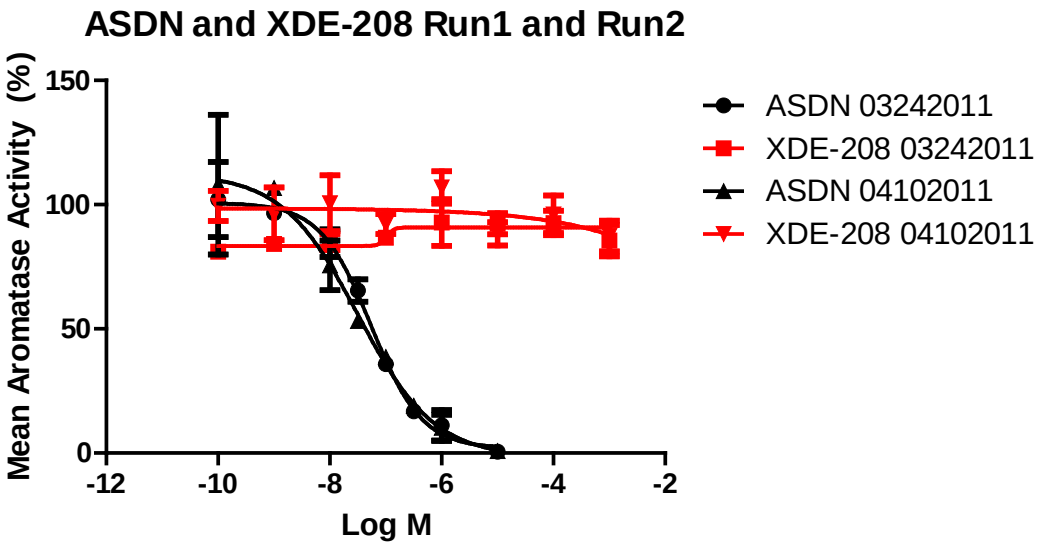


Figure 4.10.3.1.Study 9.1 (DAR Figure 6.5.4.3-4): Sulfoxaflor and ASDN– Aromatase Inhibition Combined Graph of Run 1 and Run 2.

For run 1 conducted on March 24, 2011, one data point was excluded from the graphs sulfoxaflor, 3rd assay point, concentration -7.0 LogM, leaving an n=2 for that concentration. This data was clearly an outlier and therefore removed. For run 2 conducted on April 10, 2011, 2 data points were removed from the graphs 4-OH-ASDN, -9.0 logM leaving an n=1 for that concentration, and sulfoxaflor, -7.0 leaving an n=2 for that concentration. This did not impact the data.

Conclusions

Sulfoxaflor is identified as a non-binder in the oestrogen receptor alpha (ERα) Fluorescence Polarisation (FP) assay as no displacement of the fluormone from the oestrogen receptor occurred. Sulfoxaflor is categorised as a potential binder in the androgen receptor (AR) FP assay because the binding curve exceeded the required 50% displacement of the fluormone from the receptor. The relative binding affinity of sulfoxaflor as compared to DHT was weak with a mean value of approximately 0.0005. Furthermore, the AR transactivation assays do not support the binding data identified in the FP binding assay. The AR in the FP binding assay includes only the binding domain, a truncated version of the full receptor. It is possible that the binding that occurs in the FP assay could be an event that would not occur with the full length protein (AR receptor). Other mechanisms of action could also be causing the curve to cross 50% in the FP binding assay including sulfoxaflor acting directly upon the receptor by denaturation or simply none specific binding to residues that are not normally accessible in the parent receptor molecule. None specific binding could also alter the interaction of fluormone with the AR polypeptide by changing its conformation. In two independent runs of the ERα binding assay, sulfoxaflor did not displace the fluormone from the full length oestrogen receptor and no binding occurred. The ER transactivation assay supported the binding data and sulfoxaflor is determined to not interfere with oestrogen receptor binding. Concurrent positive controls indicated specificity of the system to identify an ER binding compound.

The AR and ER transactivation assays were negative for agonism or antagonism by sulfoxaflor. Based on this, sulfoxaflor-related denaturation of the androgen receptor or other

non-specific interactions cannot be ruled out. Thus, AR-binding cannot be conclusively eliminated as a potential mechanism of action for the response observed in the AR-binding assay, though no biological effect was identified in the AR-mediated transactivation assays.

The aromatase assay determined that sulfoxaflor did not inhibit aromatase (CYP19) activity. Overall, the results from these five different *in vitro* screening tests with sulfoxaflor did not indicate changes consistent with sex steroid or classical endocrine-mediated alterations.

Study 10: Human Relevance Framework for Leydig cell Tumours. (DAR Section B.6.5.4.4.)

When all of the data described in previous sections were used in a Human Relevance Framework (HRF) analysis, the conclusion was that the observed sulfoxaflor-induced promotion of LCT in the F344 rat occurred via a dopamine enhancement MoA for which there is a moderate level of confidence. This MoA is considered not relevant to humans and supporting arguments were explained in section B.6.5.4.4; Rasoulpour et al., 2011.

Report: R. J. Rasoulpour, C. Terry, M. J. LeBaron, R. G. Ellis-Hutchings, and B. B. Gollapudi (2011). Compound: XDE-208 (Sulfoxaflor): Mode Of Action And Human Relevance Framework Analysis For XDE-208-Induced Promotion Of Fischer 344 Rat Leydig Cell Tumors. Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan, 48674. Unpublished.

Report No.: DR-0404-3134-122; Study ID: 110101.

Dates: 2011

Guidelines: Non-guideline. Not required for EU dossier submission. It is however a useful substance summary of the data regarding sulfoxaflor exposure and Leydig cell tumour incidence and relevance to man. This is submitted as a supplementary study/assessment in support of this DAR.

GLP: Not applicable.

Deviations: None. This is an acceptable overview of all the data presented thus far in section B6.5 as pertains to sulfoxaflor-induced Leydig cell tumours in rodents and the toxicological relevancy of this effect to man.

Deficiencies: None. General discussion document.

Abstract: Sulfoxaflor caused an increased size of Leydig cell tumours (LCT) at 100 and 500 ppm in a Fischer F344 Du/Crl rat carcinogenicity study. Histopathological examination confirmed that there was no increase in the overall incidence of LCT across the groups with 88, 92, 90, and 92% of rats affected at 0, 25, 100, and 500 ppm, respectively. However, there was a significant increase in bilateral LCT incidence at 500 ppm (88%) when compared to controls (64%). The background incidence of Fischer rat LCT is 75-100% in 2-year studies (88% for controls in the sulfoxaflor study) compared to 1-5% in CD rats, even less in CD-1 mice, and orders of magnitude lower in ranges of 0.01 – 0.00004% for humans. These interspecies differences in background incidence are well understood, and are the result of quantitative and qualitative differences of Leydig cell response to hormonal stimuli. Rat Leydig cells contain > 10-fold more luteinizing hormone (LH) receptors than humans, which confers greater sensitivity to slight changes in circulating LH levels. In addition to this quantitative difference, rat, but not human, Leydig cells express both prolactin receptors and gonadotropin releasing hormone (GnRH) receptors on their surface. Stimulation of rat Leydig

cells through both prolactin and GnRH receptors are a rat-specific mechanism by which LCT formation can occur. For prolactin receptor involvement in LCT, dopamine agonists (e.g., pharmaceutical class of drugs including bromocriptine) reduce prolactin release by the anterior pituitary gland eventually resulting in sustained elevations in pituitary LH release and Leydig cell stimulation and hyperplasia over a chronic duration.

Given these differences between rat and human Leydig cells, independent experts have determined "*…that human Leydig cells are quantitatively less sensitive than rat Leydig cells in their proliferative response to LH, and hence in their sensitivity to chemically induced LCTs. It can be concluded that no observable effect levels (NOELs) for the induction of LCTs in rodent bioassays provide an adequate margin of safety for protection of human health and that the data support a nonlinear mode of action (i.e., threshold response).*" Finally these experts conclude that "*...the data suggest that nongenotoxic compounds that induce LCTs in rats most likely have low relevance to humans under most exposure conditions because humans are quantitatively less sensitive than rats.*"

Analysis of the comprehensive array of available toxicology data for sulfoxaflor, including extensive non-cancer mode-of-action (MoA) data suggested a hormone-based dopamine enhancement MoA as the most likely cause of the LCT effect, which would operate through the the following key events: 1) increased neuronal dopamine release via specific dopaminergic neuron-based nicotinic acetylcholine receptor (nAChR) agonism, leading to 2) decreased serum prolactin levels, leading to 3) downregulation of LH receptor gene expression in Leydig cells, leading to 4) transient decreases in serum testosterone (T), leading to 5) increased serum LH levels, leading to 6) promotion of Leydig cell tumourigenesis. This hypothesis was evaluated in a specific MoA study in which these key events were examined to determine the causality of sulfoxaflor's promotion of Fischer rat LCT in the oncogenicity study. Additional studies were also conducted to examine whether other known potential MoAs were involved in the LCT promotion effect of sulfoxaflor. This document represents the weight of evidence approach used to evaluate the data based upon the Bradford-Hill criteria followed by subsequent application in a Human Relevance Framework (HRF).

The conclusion from this evaluation is that the LCT promotion observed in the oncogenicity study was through a subtle, but chronic, dopamine enhancement MoA in a uniquely susceptible animal model, the Fischer 344 rat. The data for sulfoxaflor are judged with a moderate degree of confidence to adequately explain the promotion of Fischer rat Leydig cell tumours following chronic dietary administration of sulfoxaflor, and judged with a very high degree of confidence to support a hormonally-mediated, threshold based, nonlinear MoA.

The promotion of Fischer rat LCT observed in the oncogenicity study has an MoA that is hormonally-mediated and threshold-based, and should be considered to have no relevance to humans due to qualitative and quantitative differences between rat and human Leydig cells. On this basis, the Fischer 344 rat Leydig cell tumours associated with lifetime administration of high dose levels of sulfoxaflor would not pose a cancer hazard to humans.

Introduction: Sulfoxaflor is a compound with insecticidal properties mediated via its agonism at the highly abundant insect nicotinic acetylcholine receptor (nAChR). In a two-year rat carcinogenicity study, male Fischer 344 rats given 100 or 500 ppm sulfoxaflor had a treatment-related increase in testis weight due to increased Leydig cell tumour (LCT) size (*Stebbins et al., 2010*). Histopathological examination confirmed that there was no increase in the overall incidence of LCT across the groups with 88, 92, 90, and 92% of rats affected at 0, 25, 100, and 500 ppm, respectively. However, there was a significant increase in bilateral

LCT incidence at 500 ppm (88%) when compared to controls (64%). In order to understand the basis for the sulfoxaflor-induced increase in Leydig cell tumour size, several mode-of-action (MoA) studies were conducted.

The analysis of the relevant toxicity and MoA studies of sulfoxaflor herein provides the context to fully evaluate the proposed MoA for LCT. This analysis is based on the specific mechanistic data generated following exposure to sulfoxaflor and indicates that the LCT promotion seen in the rat chronic/carcinogenicity study was through subtle, but chronic, enhancement of dopamine release, and subsequent inhibition of prolactin release from the pituitary gland, ultimately leading to a dopamine agonism/enhancement LCT MoA in a uniquely susceptible animal model, the Fischer 344 rat. This MoA is considered to have no relevance to humans due to qualitative and quantitative differences between human and the Fischer rat Leydig cells. In addition to providing data to support or refute specific LCT MoA, the observation of hormone level alterations in the sulfoxaflor LCT MoA study (*Rasoulpour et al., 2011*) clearly support a hormonally-mediated, and thereby threshold, nonlinear mode-of-action.

A. Relevent toxicity studies:

Below is a summary of Leydig cell tumour mode-of-action (moa)-related end points from relevant toxicity studies:

- XDE-208: Salmonella-Escherichia coli/Mammalian-Microsome Reverse Mutation Assay Preincubation Method with a Confirmatory Assay
- XDE-208: Evaluation in the Chinese Hamster Ovary Cell/Hypoxanthine-Guanine-Phosphoribosyl Transferase (CHO/HGPRT) Forward Mutation Assay
- XDE-208: Evaluation in an In Vitro Chromosomal Aberration Assay Utilizing Rat Lymphocytes
- XDE-208: Evaluation in the Mouse Bone Marrow Micronucleus Test
- XDE-208: 28-Day Dietary Toxicity Study in F344/DuCrl Rats
- XDE-208: 90-Day Dietary Toxicity Study in F344/DuCrl Rats with a 28-Day Recovery Phase
- XDE-208: Two-Year Chronic Toxicity/Oncogenicity Study in F344/DuCrl Rats
- XDE-208: Two-Generation Dietary Reproductive Toxicity Study in Crl:CD (SD) Rats
- XDE-208: Oncogenicity Study in Crl:CD1(ICR) Mice
- XDE 208: Leydig Cell Mode-of-Action Study in Crl:CD(SD) and F344/DuCrl Rats
- XDE-208 Technical Screening for Estrogen Receptor and Androgen Receptor Binding and Transactivation and Aromatase Inhibition

B. Background on leydig cell tumour incidence and relevance to humans:

The toxicology relating to Leydig cell tumourigenesis in rats and its human relevance has been reviewed extensively (*Cook et al., 1999; Clegg et al., 1997; Prentice and Miekle, 1995*). These LCTs initially appear as hyperplasia of interstitial cells that can grow with

age to the diameter of a single normal seminiferous tubule, at which point they are classified as adenomas per guidance from the National Toxicology Program (NTP) (*Boorman et al., 1990; Boorman et al., 1987*).

Leydig cell tumour background incidence across strains/species

The high background incidence of LCTs in Fischer 344 rats has been well-known for decades with spontaneous adenomas commonly present at 12 months and their incidence increasing to 75-100% by 24 months (*Boorman et al., 1990*). In contrast, the CD rat has a background incidence of 1-5% at 24 months, while CD-1 mouse incidences are even lower at <1-2.5% (*Cook et al., 1999*). With regards to human relevance, estimates of human LCTs are orders of magnitude lower with ranges of 0.01 – 0.00004% incidence (*Cook et al., 1999; Mati et al., 2002*). Table 6.5.4.4-1 summarises these species and strain differences.

Table 4.10.3.1.Study 10.1 (DAR Table 6.5.4.4-1) Species/Strain Background Incidence of Leydig Cell Tumours

Species	Strain	Background Incidence of LCT
Rat	F344	75-100%
	Wistar	6%
	CD	5%
Mice	CD1	2.5%
	B6C3F1	0.4%
Human		0.00004-0.01%

Molecular basis of the difference in species/strain incidence of LCTs

Given the strong qualitative similarities in the hypothalamic-pituitary-gonadal (HPG) axis (Figure 6.5.4.4-1) among rats (Fischer and CD), mice, and humans, the stark difference in prevalence of LCT, especially between F344 rats and humans, indicates that these interspecies differences are due to quantitative differences in Leydig cell response to stimuli via LH and GnRH receptors. Qualitative differences also exist between rats and humans. Specifically, much of the testosterone (T) in human serum is bound to steroid binding protein, whereas in rodents T circulates as free hormone and is more easily conjugated and metabolised. This difference makes rodents more susceptible to alterations in plasma T levels.

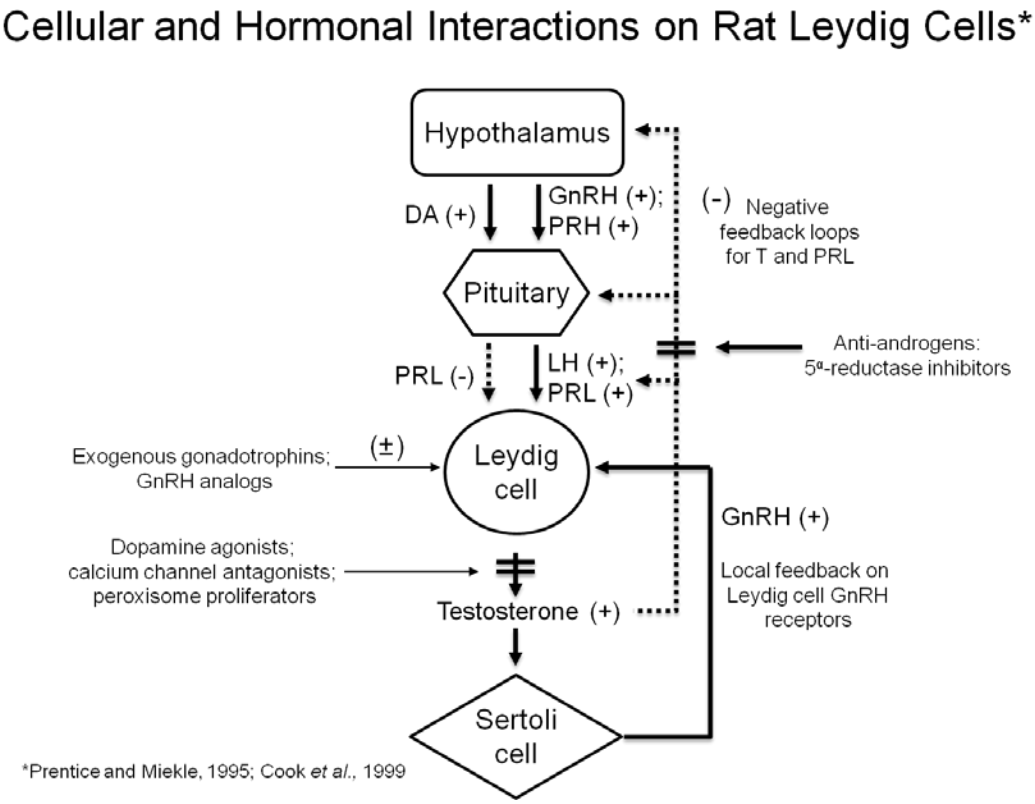


Figure 4.10.3.1.Study 10.1 (DAR Figure 6.5.4.4-1): The hypothalamic-pituitary-gonadal (HPG) axis among rodents.

In both rodents and humans, LH stimulates Leydig cells to produce testosterone; however, rat Leydig cells have 20,000 LH receptors compared to only 1,500 LH receptors in human Leydig cells (*Huhtaniemi, 1983*). This > 10-fold higher number of LH receptors in the rat confers a far greater sensitivity to slight changes in LH levels, compared to the relatively unresponsive human Leydig cell. It is due to the large number of 'spare' receptors in the rat that LH receptor occupancy of only 1% is sufficient to elicit a signal transduction cascade response, which confers the greater sensitivity in rats to slight changes in LH levels (*Katzung, 1995*).

In addition to a greater LH receptor density, rat, but not human, Leydig cells have GnRH receptors (*Clayton and Huhtaniemi, 1982*) and prolactin receptors on their surface (*Cook et al., 1999*). Therefore, stimulation of rat Leydig cells through these receptors is a rat-specific mechanism by which LCT induction can also occur. For GnRH receptors, this position is supported by the fact that GnRH agonists such as buserelin can induce LCTs in rats through the pituitary gland and direct activation at the Leydig cell, but at high doses can suppress testosterone via inhibition of LH release through negative feedback at the level of the pituitary gland (*Donabauer et al., 1987; Negro-Vilar and Valenca, 1988*). For prolactin receptor involvement in LCTs, dopamine agonists, such as muselergine, reduce prolactin release by the anterior pituitary gland, which results in a decreased binding to prolactin receptors on Leydig cells (*Prentice and Miekle, 1995*). This decreased prolactin receptor stimulation results in downregulation of LH receptors, and therefore lower testosterone levels, which feeds back to induce LH release from the pituitary leading to Leydig cell stimulation and hyperplasia (*Prentice et al., 1992*).

Human relevance

As summarised here and reviewed extensively elsewhere (*Cook et al., 1999; Mati et al., 2002*), LCTs in rats can be induced through alteration at the HPG axis resulting in excessive stimulation of Leydig cells, with Fischer 344 rats having almost 100% prevalence of this tumour type by 24 months of age. This is 10,000-1,000,000 times higher than published human incidences of this tumour type (*Cook et al., 1999; Mati et al., 2002*). Research into differences between rat and human Leydig cells supports this epidemiological data: rat Leydig cells are more responsive to testosterone homeostasis perturbations due to a higher number of LH receptors and the presence of prolactin and GnRH receptors on the cell surface.

Taken together, experts have independently determined that "*…human Leydig cells are quantitatively less sensitive than rat Leydig cells in their proliferative response to LH, and hence in their sensitivity to chemically induced LCTs. It can be concluded that no observable effect levels for the induction of LCTs in rodent bioassays provide an adequate margin of safety for protection of human health and that the data support a nonlinear mode of action (i.e., threshold response).*" Finally these experts conclude that "*…the data suggest that nongenotoxic compounds that induce LCTs in rats most likely have low relevance to humans under most exposure conditions because humans are quantitatively less sensitive than rats.*" (*Cook et al., 1999*).

C. Modes of action for rodent Leydig cell tumours:

It is generally accepted in the literature that there are 9 known modes-of-action for Leydig cell tumour induction in rats, which fall into three 'bins' of human relevance (i.e., relevant, low relevance, no relevance; *Cook et al., 1999*). These are:

Relevant to humans:	(1) mutagenicity
Low relevance to humans:	(2) androgen receptor antagonism
	(3) oestrogen receptor agonism/antagonism
	(4) 5-alpha-reductase inhibition
	(5) aromatase inhibition
	(6) **reduced testosterone biosynthesis**
	(7) **increased testosterone biliary elimination**
No relevance to humans:	(8) GnRH (LHRH) agonism
	(9) **Dopamine agonism/enhancement**

Detailed explanations of these MoAs are described elsewhere (*Cook et al., 1999*), but apart from MoA #1 (mutagenicity), all operate via a hormonally-mediated, threshold-based, MoA where they induce a sustained increase in circulating LH levels, thereby causing a trophic stimulation of Leydig cells leading to hypertrophy/hyperplasia and ultimately LCTs; thus, it is important to identify key early events in this process to conclusively demonstrate the specific MoA for sulfoxaflor. As stated previously, the inherent difference in responsivity of rat *vs.* human Leydig cells confers quantitatively low human relevance for all known non-mutagenic LCT MoAs. In addition, the presence of GnRH and prolactin receptors on rat, but not human, Leydig cells confers partial qualitative differences (i.e., no relevance) for MoA #8 and #9

(GnRH agonism and Dopamine agonism/enhancement, respectively), making them of no relevance to humans.

The proposed MoA for sulfoxaflor promotion of Fischer rat LCT is MoA #9, which will be discussed in more detail here. An alternative MoA analysis for sulfoxaflor is presented later in this document.

Key events for MoA #9 (dopamine agonism/enhancement).

Within MoA #9, the catecholamine neurotransmitter dopamine (also known as prolactin inhibitory factor) is released from the hypothalamus and travels via the hypothalamic-hypophyseal portal system to the anterior pituitary gland where it directly inhibits release of prolactin hormone into systemic circulation (*Casarett et al., 2007*). Higher serum prolactin levels causes downregulation of LH receptors (LHR) within the rat Leydig cells (*Prentice et al., 1992*). Decreased LHR gene expression results in slight decreases in testosterone production, which feeds back to the hypothalamus and pituitary gland to cause a compensatory increase in circulating LH to maintain testosterone at physiological concentrations (*Cook et al., 1999*). As with all hormone-based, threshold mechanisms of rodent Leydig cell tumourigenesis, the compensatory increase in LH levels leads to increased Leydig cell proliferation and tumours.

As a number of the rodent LCT MoAs have common hallmarks of changes in LH and testosterone levels, it is important to pay particular attention to the components of a particular MoA that would help clearly distinguish between early changes. In the case of MoA #9, the unique key events are an increase in dopamine within the hypothalamic-hypophyseal portal system, decrease in circulating serum prolactin levels, and a decrease in LHR gene expression within the testis. Unfortunately, measuring neurotransmitter levels within the portal system between the hypothalamus to the anterior pituitary is an extremely difficult procedure. Fortunately, inhibition of prolactin secretion is primarily dependent on dopamine signaling; therefore, a decrease in circulating serum prolactin levels, which is easily measured, is an appropriate indirect measure of the direct effect of neuronal dopamine enhancement and also a unique identifying feature of MoA #9. Therefore, when identifying early key events responsible for a dopamine agonism/enhancement MoA, the most important indicators of causality of this are lower serum prolactin and LHR gene expression within the testis. Other measurements, such as increased LH and decreased testosterone, while directly related to a hormonally-mediated MoA, are simply supportive and common to many other LCT MoAs. The Key Events for assessment of the dopamine agonism/enhancement MoA are described in detail below, and are also listed in Table 6.5.4.4-2.

Table 4.10.3.1.Study 10.2 (DAR Table 6.5.4.4-2). Key Events for Dopamine Agonism/Enhancement MoA

(1)	Increased dopamine release
(2)	Decreased serum prolactin levels
(3)	Downregulation of LH receptor gene expression in Leydig cells
(4)	Transient decrease in serum testosterone levels
(5)	Increased serum LH levels
(6)	Promotion of Leydig cell tumourigenesis

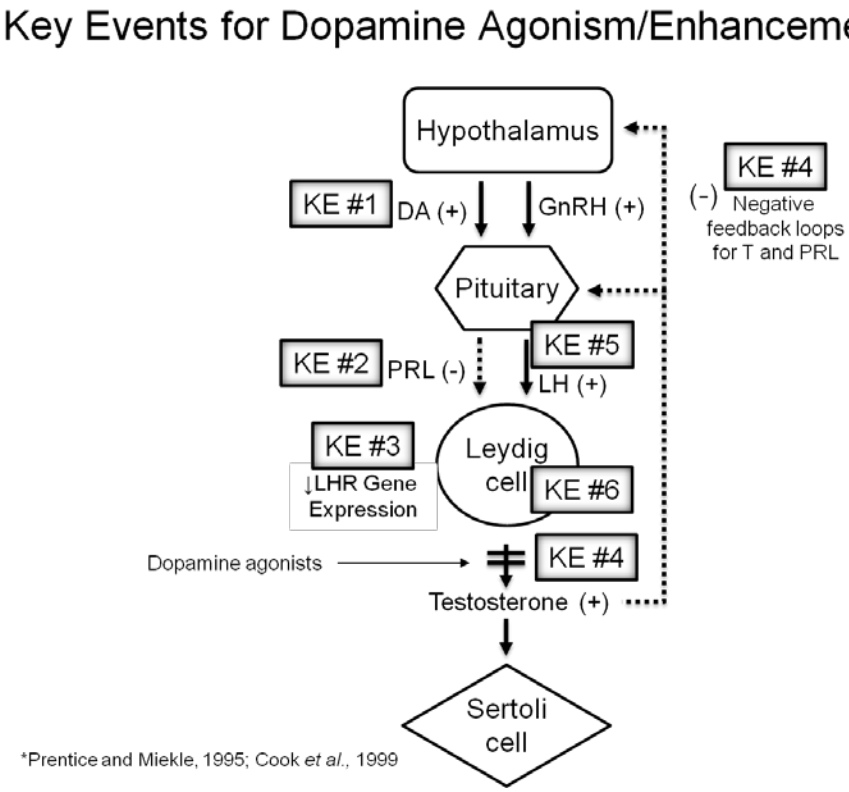


Figure 4.10.3.1.Study 10.2 (DAR Figure 6.5.4.4-2): The key events in rodents that are thought to lead to Leydig cell hypertrophy, hyperplasia and eventual tumourigenesis.

Key Event #1: Increased dopamine release

Release of dopamine from the arcuate nucleus into the median eminence and hypophysial portal vessels is the first key event in MoA #9. Due to complexity and size of these structures, it is difficult to directly measure alterations in dopamine secretion; stereotaxic implantation of a cannula within the lateral cerebroventricle would be required to measure potential changes in dopamine release. As standard practice, inhibition of serum prolactin levels are routinely used as an indirect measure of dopamine release, although a decrease in Prl secretion is directly mediated by dopamine agonism on the Prl-producing cells (lactotrophs) in the anterior pituitary (*Ben-Jonathan and Hnasko, 2001*).

Key Event #2: Decreased serum prolactin levels

Increased dopamine release from the hypothalamus to the anterior pituitary directly inhibits the release of prolactin from the pituitary into the systemic circulation. As dopamine is the primary negative regulator of prolactin levels, a decrease in circulating prolactin levels is most likely resultant from an increase in dopamine secretion (*Ben-Jonathan and Hnasko, 2001*), which is the mechanism by which FDA approved pharmaceutical dopamine agonists (e.g., bromocriptine) treat hyperprolactinemia.

Key Event #3: Downregulation of LH receptor gene expression in Leydig cells

As mentioned previously, rat Leydig cells contain prolactin receptors, which are involved in maintaining expression of LH receptors (LHR). Lower circulating prolactin levels lead

to decreases in prolactin binding of the prolactin receptor on Leydig cells, which, in turn, leads to downregulation of LHR gene expression (*Williams et al., 2007; Prentice et al., 1992*).

Key Event #4: Transient decrease in serum testosterone levels

In response to downregulation of LHR gene expression, there is a transient dip in serum testosterone levels, which provides negative feedback to the hypothalamus and pituitary gland (*Cook et al., 1999*). Data from the dopamine agonist mesulergine in rats demonstrates no hormone changes after 2 weeks, a transient dip at 4 weeks, no effect at 10 weeks, and then an increase in testosterone at 13 weeks (*Prentice et al., 1992*). These data demonstrate that even with clear pharmaceutical-designed dopamine agonists, the feedback loops within the HPG axis make hormone changes difficult to measure with an MoA study. In addition, hormone levels often fluctuate as a result of subtle diurnal or pulsatile variations that may confound interpretation; however, longterm alteration of the HPG axis (or other) may result in an apical alteration attributed to subtle hormonal changes.

Key Event #5: Increased serum LH levels

Common to the numerous hormone-based LCT MoAs is an increase in LH being the causative agent for Leydig cell proliferation (*Cook et al., 1999*). In the case of MoA #9, this increase in serum LH levels is due to compensation for the transient dip in testosterone levels (KE #4), which feeds back to the level of the hypothalamus and pituitary leading to increased LH release.

Key Event #6: Promotion of Leydig cell tumourigenesis

The final key event/apical end point is an increase in Leydig cell proliferation and/or size of the LCTs. It is important to note that the difference between the histopathological classification of Leydig cell hyperplasia versus Leydig cell tumours is simply based on the size of the cluster of Leydig cells. In the case of hyperplasia, the mass of Leydig cells are smaller than the diameter of a single normal seminiferous tubule. When the proliferative interstitial cells reach a diameter of greater than a single normal seminiferous tubule, they are classified as adenomas, per guidance by the National Toxicology Program (NTP).

D. Sulfoxaflor rodent leydig cell tumour postulated mode of action (MoA):

The relevant experimental data for evaluation of the sulfoxaflor-induced rodent LCT MoA and human relevance includes guideline short-term/sub-chronic studies in the rat (28-day and 90-day), the two-generation reproductive toxicity study in rats, oncogenicity studies in the rat and mouse, as well as specific *in vivo* and *in vitro* Leydig cell MoA studies. These studies will be presented in more detail during the evaluation of the MoA. During the MoA analysis, it is important to note that the apical end point findings are an increase in Leydig cell tumour size in Fischer 344 rats given 100 or 500 ppm sulfoxaflor for two years. The extremely high background incidence of LCT in control Fischer rats at this age (historical range 75-100%; 88% for controls in the sulfoxaflor study) is indicative of the unique biology of this strain of rat (*Cook et al., 1999*). Therefore, for hormone-based MoAs, one would expect only subtle changes in young animals during shorter durations of exposure as the apical end point of increased Leydig cell tumour size results from a combination of the testis biology in a senescent Fischer rat and promotion of this normal biological process by sulfoxaflor exposure. The hypothesised key events for the

sulfoxaflor-induced rodent Leydig cell tumours have been listed in table 6.5.4.4-2, and the data that support these key events are described in subsequent sections in this document.

Key Event #1: Increased Dopamine Release via nAChR Agonism

The release of dopamine via central nAChR agonism by sulfoxaflor has not been tested directly due to the inherent complexity of the biology, and technical challenges of measuring local releases of a neurotransmitter in this system. However, because inhibition of prolactin release (Key Event #2) is primarily driven by dopamine release, the connection has been established with pharmaceutical dopamine agonists (*Prentice et al., 1992*), and this endpoint can be used as indirect evidence that sulfoxaflor causes this key event. This section describes the complexity and relationship between neuroendocrine dopamine levels and nAChRs.

Dopamine is a catecholamine neurotransmitter associated with reward centers of the brain. Primary types of dopaminergic neurons in the adult rat brain exist within the:

1) dorsal (nigrostriatal) pathway originating in the substantia nigra and terminating in the caudate-putamen

2) ventral (mesolimbic) pathway originating in the ventral tegmental area and terminating in the nucleus accumbens

3) neuroendocrine pathway originating in the arcuate nucleus and terminating in the median eminence (*Gianoulakis, 1998*).

This third type of dopaminergic neuron pathway is relevant for the LCT MoA #9 as it is the pathway responsible for dopamine release at the median eminence into the hypothalamic-hypophyseal portal veins to inhibit prolactin release in the anterior pituitary (*Gianoulakis, 1998; Casarett et al., 2007*).

Central nicotinic acetylcholine receptors (nAChRs), such as α4β2 and α4α6β2 nAChRs, play a key regulatory role in dopamine release from dopaminergic neurons in the brain (*Maskos, 2010*). Unfortunately, the nAChR subunit characterization within the rat arcuate nucleus and median eminence is the least characterised (Neil Millar, University College London, and Susan Wonnacott, University of Bath personal communications) and limited to immunohistochemistry of α4 subunits in neuronal axons of the median eminence (*Okuda et al., 1993*). Unfortunately these data are of limited value as there are multiple different α4-containing nAChRs involved in dopamine-release in pathways 1 and 2. Due to the lack of characterisation of the nAChR subunits in this area of the brain, *in vitro* recombinant receptor agonism studies were not performed because it would not have been possible to place the findings within context of the *in vivo* system. Most of the available literature connecting central nAChRs and dopamine regulation come from pathways 1 and 2 (above), which are used as surrogates here to establish plausibility.

Microinjection of cholinergic agonists in the substantia nigra pars compacta, a brain region containing dopminergic neurons, dose-dependently increased dopamine efflux (*Blaha and Winn, 1993*). Partial agonists to the α4β2 nAChR have been used as smoking cessation drugs (e.g., Tabex or cytisine) by causing release of dopamine in smaller portions to compensate for nicotine withdrawal (*Cassels et al., 2005*). The insecticidal MoA for sulfoxaflor is binding to and agonism on the insect nAChR, and it has been shown that

sulfoxaflor is also an agonist to the rat foetal muscle subtype of the nAChR (*Rasoulpour et al., 2011*). It is plausible that the LCT promotion seen in the rat chronic/carcinogenicity study was through subtle, but prolonged, agonism at the central nAChRs within the median eminence causing release of dopamine and inhibition of prolactin release from the pituitary gland.

Key Event #2: Decreased Serum Prolactin Levels

In direct response to dopamine release from the hypothalamus to the anterior pituitary gland (Key Event #1), prolactin secretion to the systemic circulation is inhibited. Levels of serum prolactin were measured in the LCT MoA study (section B.6.5.4.1; *Rasoulpour et al., 2010*) at 2, 4, and 8 weeks of exposure to 0, 25, 100, or 500ppm sulfoxaflor in Fischer rats. There was no effect of sulfoxaflor treatment on serum prolactin levels after two weeks of treatment; however, there was a 1.7-fold decrease in serum prolactin at 4 weeks in the 500ppm group with a concomitant 2-fold increase in serum LH levels (see Key Event #5), as shown originally in table 6.5.4.1-3 and figure 6.5.4.4-3 below. The effect on prolactin levels was not observed at the 8-week time point, which suggests compensation by the HPG axis. Note that the increase in baseline prolactin levels from 2-8 weeks in controls seen in this study is typical for young male rats (*Prentice et al., 1992*). Terminal blood samples were also collected from this LCT MoA study; however, because prolactin is a stress related hormone, levels across all groups were induced in response to carbon dioxide euthanasia associated stress: being 3-5-fold higher than in-life bleeds – the data can be seen in table 6.5.4.1-4 from section B.6.5.4.1. Table 6.5.4.4-3 shows the temporal and dose response for decreased prolactin levels from the LCT MoA study.

Table 4.10.3.1.Study 10.3 (DAR Table 6.5.4.4-3) Sulfoxaflor Key Event #2: temporal and dose response for decreased serum prolactin levels.

Temporal →

Dose ↓

Dose ppm	2 weeks	4 weeks	8 weeks
0	100	100	100
25	135	86.5	92.8
100	86.1	90.4	100
500	88.8	**58.1**	90.6

Data are percentage of control values. **Bold** indicates treatment-related.

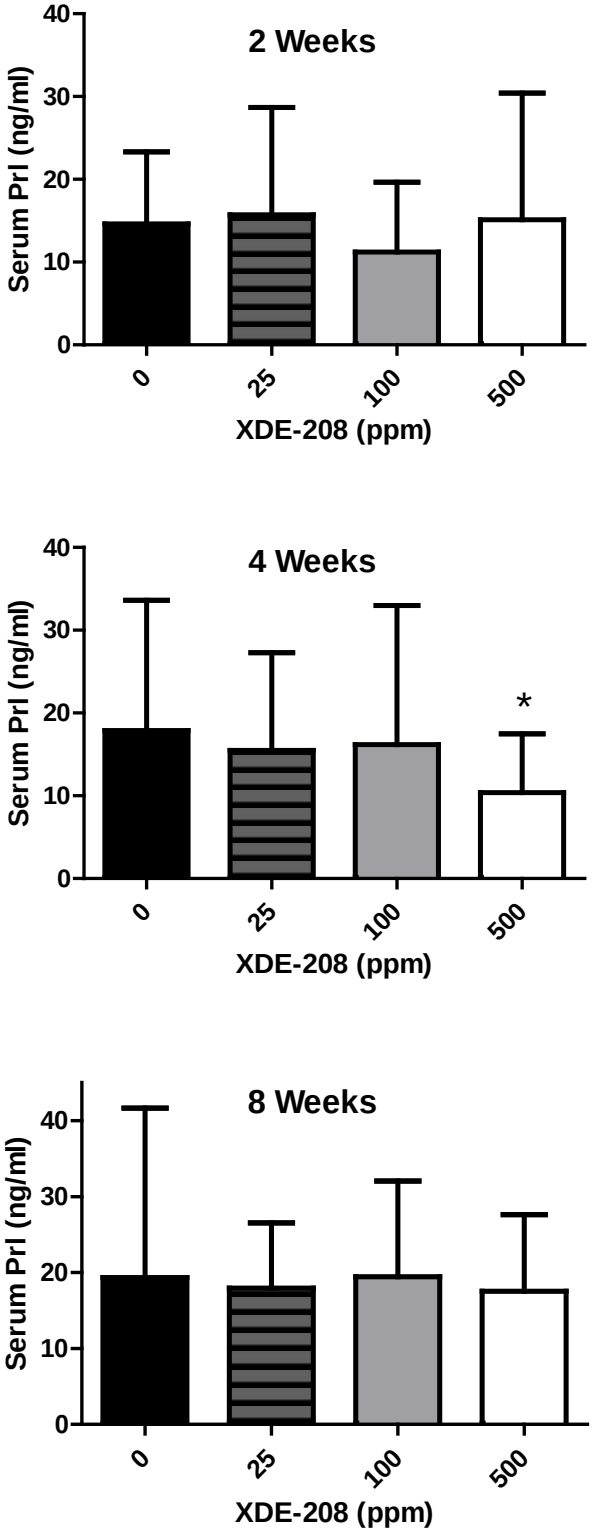


Figure 4.10.3.1.Study 10.3 Figure 6.5.4.4-3. Sulfoxaflor: Fischer Rat Serum Prolactin Levels (Mean ± S.D.) upon treatment with sulfoxaflor. Sampling time points were 2, 4, and 8 weeks from start of dosing regime.

While subtle and transient, the prolactin hormone data provide support for MoA #9 (dopamine agonism/enhancement) with the key signature of a decrease in Prl levels apparent.

This would only be observed with MoA #9 and would not be associated with the other possible mechanisms leading to Leydig cell tumours (LCT). Furthermore the decrease in Prl levels were associated with a compensatory increase in LH levels (table 6.5.4.4-9), which in turn acted as the primary trophic stimulus over the two-year Fischer rat carcinogenicity study leading to LCT promotion. The additional concordance of a slight increase in testosterone with increased LH levels at 4-weeks supports that this LH increase is a biologically meaningful change in Fischer rats (tables 6.5.4.4-7 and 6.5.4.4-9). Due to the persistent compensatory nature of the hypothalamic-pituitary-gonadal (HPG) axis, coupled with the fact that chronic sulfoxaflor exposure for two years was required for increased LCT size (and bilateral, but not unilateral, incidence) in Fischer rats, it is not surprising that the changes observed in the hormone data are temporal in nature. In general for hormone-based MoAs, one would expect only subtle changes in young animals during shorter durations of exposure as the apical end point of increased Leydig cell tumour size results from a combination of the testis biology in a senescent Fischer rat and promotion of this normal biological process by sulfoxaflor exposure. This interpretation is supported by the fact that conclusive sulfoxaflor Leydig cell effects in the guideline toxicity studies occurred only at the two-year time point. These hormone changes, in addition to providing data to support or refute specific MoAs for LCT, support a hormonally-mediated, and thereby threshold, nonlinear mode-of-action.

Key Event #3: Downregulation of LH Receptor Gene Expression in Leydig Cells

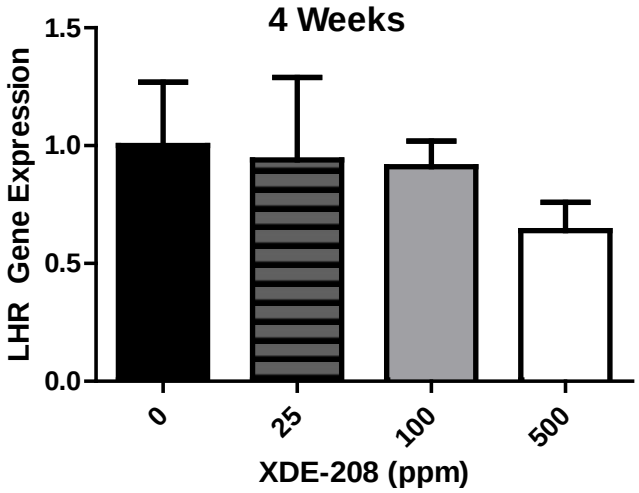
In Key Event #3 of the dopamine agonism/enhancement MoA, lower serum prolactin levels (Key Event #2) in rats, but not humans, would lead to downregulation of LH receptor (LHR) gene expression (*Williams et al., 2007; Prentice et al., 1992*). Lower LHR expression would lead to a transient dip in testosterone production, leading to HPG-axis feedback stimulation and ultimately to increased LH release. Therefore if MoA #9 were operant, LHR gene expression would be decreased consistent with decreased circulating Prl hormone and increased LH. Real-time PCR was performed on 4- and 8-week isolated Fischer rat testis mRNA for the LH receptor (LHR) and prolactin receptor (PrlR) genes in order to determine if there was molecular concordance to the hormone data, which supported the dopamine enhancement MoA (section B.6.5.4.1; *Rasoulpour et al., 2010)*.

Consistent with MoA #9 and the decreased Prl levels in the 4-week Fischer rat hormone data in Key Event #2, there was a ~1.6-fold dose-dependent decrease in LHR gene expression at the 4-week, but not 8-week, time point (table 6.5.4.4-4 and figure 6.5.4.4-4). In addition, there was a decrease in PrlR gene expression at the 4-week, but not 8-week, time point. While not statistically significant the magnitude of gene expression changes is consistent with the dynamic range of these genes *in vivo* and likely represents a biologically meaningful effect based on alterations in hormone levels. This conclusion is supported by a recent publication where administration of exogenous Prl to rats for 4-weeks resulted in a ~2-fold increase in LHR gene expression (*Williams et al., 2007*).

Consistent with the decrease in serum prolactin observed after 4-weeks of treatment with 500ppm sulfoxaflor, there was a biologically significant decrease in LHR gene expression at this dose level and time point. Also consistent with the prolactin hormone data were no differences from control of any other treatment group for LHR gene expression. Table 6.5.4.4-5 shows the temporal and dose response for downregulation of the LHR from the LCT MoA study.

Table 4.10.3.1.Study 10.4 (DAR Table 6.5.4.4-4.) Fischer Rat Testis LHR Gene Expression	
Dose ppm	**LHR**
4-Week Treatment	
0	1
25	-1.1
100	-1.1
500	**-1.6**

8-Week Treatment	
0	1
25	-1.3
100	1.2
500	1.1
Bold type indicates a treatment-related effect Data presented as + or - fold-change of control	



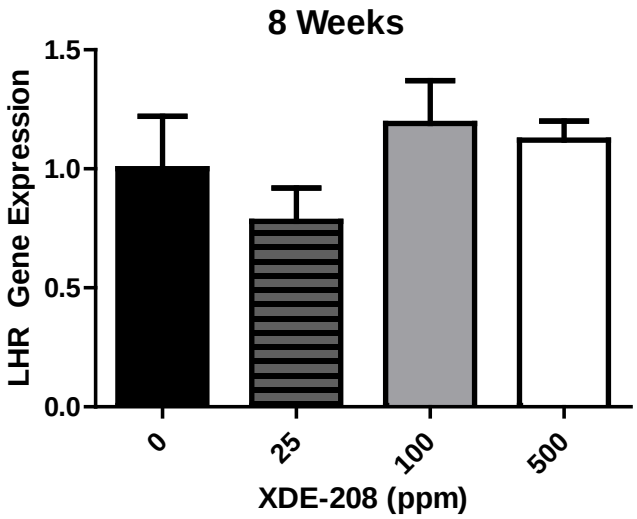


Figure 4.10.3.1.Study 10.4 (DAR Figure 6.5.4.4-4.) Sulfoxaflor: Fischer Rat Testis LHR Gene Expression (Mean ± S.D.).

Table 4.10.3.1.Study 10.5 (DAR Table 6.5.4.4-5.) Sulfoxaflor Key Event #3: Temporal and Dose Response for Decreased Fischer Rat Testis LHR Gene Expression

Temporal →

Dose ↓

Dose ppm	4 weeks	8 weeks
0	100	100
25	94	78
100	91	119
500	**64**	112

Bold type indicates a treatment-related effect

Data are percentage of control values

Key Event #4: Transient Decrease in Serum Testosterone Levels

Downregulation of the LHR in Key Event #3 leads to a transient decrease in serum testosterone levels in Key Event #4 (*Cook et al., 1999*). In LCT MoA experiments with the dopaminergic pharmaceutical agent mesulergine, serum testosterone levels were similar to controls at 2-weeks of treatment, slightly lower than controls at 4 weeks, returned to baseline by 10 weeks, and were elevated at 13 weeks (*Prentice et al., 1992*). Within the sulfoxaflor LCT MoA study, there were no measured decreases in serum testosterone levels at the 2-, 4-, or 8-week time point, table 6.5.4.4-7. However, in the two-generation reproductive toxicity study (see section B.6.6; *Rasoulpour et al., 2010*), there was a treatment-related delay in balanopreputial separation (BPS) for male offspring in the high-dose group of 400ppm

sulfoxaflor, summarised in table 6.5.4.4-6 below. The process of BPS as a pubertal onset marker in a male rat is dependent on androgen levels, as testosterone injection to castrated rats is sufficient to induce BPS (*Korenbrot et al., 1977*). Therefore, in order for sulfoxaflor to induce a delay in BPS within the two-generation reproductive toxicity study, there had to be a decrease in testosterone levels (for at least some duration) during postnatal development. Further support for this statement is the fact that dopamine agonists such as bromocriptine induce a delay in male rat BPS (*Marty et al., 2001*).

This decrease in testosterone levels leading to a delay in BPS must have been a transient event as there were no effects on accessory sex gland weight, histopathology, or any other anti-androgenic finding in the adult males within the two-generation reproductive toxicity study that had a delay in BPS. While many anti-androgenic molecules can cause a delay in BPS, these direct acting anti-androgens also cause a shortening in anogenital distance (AGD) at birth (*Wolf et al., 2000*). Interestingly, there was no effect on AGD within the two-generation study on sulfoxaflor, which is also consistent with a

Table 4.10.3.1.Study 10.6 (DAR Table 6.5.4.4-6.) Crl:CD(SD) Rat Balanopreputial Separation

Dose ppm	Pubertal Parameter
Age at Attainment (days)	
0	44.6
25	46.4
100	44.5
400	**47.0***
Body Weight at Attainment (g)	
0	253.6
25	265.8
100	250.3
400	**272.8**
* Statistically different from control mean by Dunnett's test, alpha = 0.05 **Bold type** indicates treatment-related effect	

Table 4.10.3.1.Study 10.7 (DAR Table 6.5.4.4-7.) Fischer Rat Serum Testosterone Levels

Dose ppm	Serum Testosterone (ng/g)
2-Week Treatment	
0	0.76
25	0.83
100	0.54
500	0.90
4-Week Treatment	
0	0.67
25	1.00
100	1.19
500	0.93

8-Week Treatment	
0	0.58
25	0.67
100	0.77
500	0.70

dopamine agonist/enhancer MoA as maternal prolactin levels during gestation are sufficient to abrogate any prolactin decrease effect in perinatal male rats (*Ben-Jonathan and Hnasko, 2001*). Table 6.5.4.4-8 shows the temporal and dose response for the key event of transient decrease in serum testosterone levels.

Dose

Table 4.10.3.1.Study 10.8 (DAR Table 6.5.4.4-8.) Sulfoxaflor Key Event #4: Transient Decrease in Serum Testosterone Levels	
Dose ppm	**Decreased T as assessed by BPS in 6-7 wk old males**
0	-
25	-
100	-
400	↓*
(-) = no change versus control; *indicates indirect data from delay in balanopreputial separation data	

Temporal

Dose

Dose ppm	**2 weeks**	**4 weeks**	**8 weeks**
0	100	100	100
25	109	149	116
100	71	178	133
500	118	139	121
Data are percentage of control values.			

Key Event #5: Increased Serum LH Levels

Common to most hormone-based LCT MoAs is an increase in serum LH acting as the causative agent for providing trophic stimulus of Leydig cells towards hyperplasia and eventually adenomas (*Cook et al., 1999*). The dopamine agonism/enhancement MoA is no exception to an eventual increase in LH leading to LCTs. With respect to sulfoxaflor, there was a dose-dependent increase in serum LH levels at the 4-week timepoint in Fischer rats (table 6.5.4.4-9 and figure 6.5.4.4-5), consistent with timing of decreased prolactin level, which was observed in the LCT MoA study.

Terminal blood samples were also collected from this LCT MoA study; however, as LH is a

pulsitile and stress affected hormone, levels across all groups were induced ~8-10-fold higher than in-life bleeds in response to carbon dioxide euthanasia-associated stress – the complete data can be seen in table 6.5.4.1-4 from section B.6.5.4.1.

Table 4.10.3.1.Study 10.9 (DAR Table 6.5.4.4-9.) Fischer rat serum LH levels

Dose ppm	Serum LH (ng/ml)
2-Week Treatment	
0	0.54
25	0.81
100	0.27
500	0.42
4-Week Treatment	
0	0.47
25	0.54
100	0.66
500	**0.88**
8-Week Treatment	
0	0.89
25	1.04
100	0.69
500	1.08
Bold type indicates treatment-related effect	

Table 4.10.3.1.Study 10.10 (DAR Table 6.5.4.4-10.) Key Event #5: temporal and dose response for serum LH levels

Temporal 

Dose

Dose ppm	2 weeks	4 weeks	8 weeks
0	100	100	100
25	150	114	116
100	50	140	77
500	78	**187**	121

Data are percentage of control values. **Bold** indicates treatment-related.

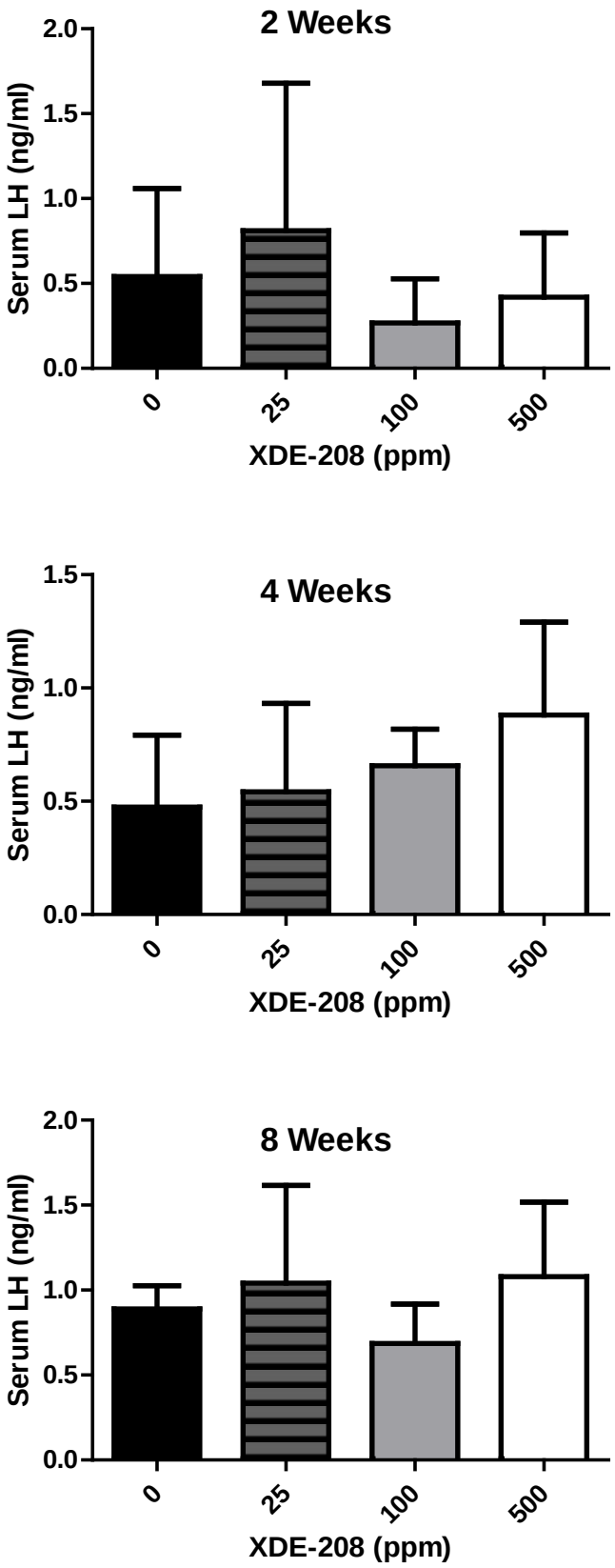


Figure 4.10.3.1.Study 10.5 (DAR Figure 6.5.4.4-5.) Sulfoxaflor: Fischer Rat Serum LH Levels (Mean ± S.D.)

As outlined in table 6.5.4.2/3-10, there was no effect of treatment on Fischer rat hormone

levels at the 2- or 8-week timepoints; however, at 4-weeks there was an ~1.9-fold dose-dependent increase in LH levels concomitant with a ~1.7-fold dose-dependent decrease in prolactin levels.

Due to the persistent compensatory nature of the hypothalamic-pituitary-gonadal (HPG) axis, coupled with the fact that chronic (i.e., two years) sulfoxaflor exposure was required for increased LCT size in Fischer rats, it is not surprising that the changes observed in the hormone data are temporal in nature. A conclusion supported by the fact that conclusive Leydig cell hyperplastic effects in the guideline toxicity studies occurred only at the two-year time point.

Key Event #6: Promotion of Leydig Cell Tumourigenesis

In a rat chronic/carcinogenicity study, Fischer 344 rats were given 0, 25, 100, or 500ppm sulfoxaflor for 24 months (see section B.6.5.1.1; *Stebbins et al., 2010*). There was a treatment-related increase in paired testis weight at 100 and 500ppm that was due to an increased size of Leydig cell tumours (LCT) in these animals (table 6.5.4.4-11). Histopathological results confirmed that there was no increase in the overall incidence of LCT across the groups with 88, 92, 90, and 92% of male rats with these tumours at 0, 25, 100, and 500ppm, respectively. However, there was a significant increased incidence of animals with bilateral LCT at 500ppm.

Before these findings were observed at the two-year time point of the rat chronic/carcinogenicity study, the only related effect was a slight 2.4-day delay in balanopreputial separation (BPS) at 400ppm in the two-generation study in Crl:CD(SD) rats. At the one-year time point in the rat oncogenicity study, there were 0, 1, 3, and 3 LCT at the 0, 25, 100, and 500ppm dose groups, which was deemed unrelated to treatment because this was within the historical control range (0-3 LCT at one-year) and a lack of a dose-response between 100 and 500 ppm at this time point. Presented in table 6.5.4.4-12 is the temporal and dose reponse for increased Leydig cell proliferation/size of tumours.

Table 4.10.3.1.Study 10.11 (DAR Table 6.5.4.4-11.) Sulfoxaflor: Two-year Fischer rat testes weights.

Dose (ppm)	Final Body Weight (g)	Testes Weights (g)	Testes Weights (g/100)
Two-Year Treatment			
0	415.2	3.720	0.906
25	418.4	3.933	0.940

100	**396.0**	**5.423***	**1.359***
500	**394.2**	**6.025***	**1.519***

Bold type indicates treatment-related effect

***** Statistically different from control mean by Dunnett's test, alpha = 0.05

Table 4.10.3.1.Study 10.12 (DAR Table 6.5.4.4-12.) Sulfoxaflor Key Event #6: temporal and dose response for promotion of Leydig cell tumourigenesis

Temporal →

Dose ↓

Dose ppm	<52 weeks	52 weeks	104 weeks
0	-	-	-
25	-	-	-
100	-	-	+
500	-	-	+
+ indicates effect present, - indicates effect absent.			

Table 4.10.3.1.Study 10.13 (DAR Table 6.5.4.4-13.) Temporality and dose response for MoA key events related to male F344 rat Leydig cell tumours

Temporal →

Dose ↓

Dose (ppm)	Key Event 1	Key Event 2	Key Event 3	Key Event 4	Key Event 5	Key Event 6
	Increased dopamine release via nAChR agonism	Decreased serum prolactin levels	Downreg of LHR gene expression in Leydig cells	Decreased serum testosterone levels	Increased serum LH levels	Promotion of Leydig cell tumours
25		-	-	-	-	-
100		-	-	-	-	+
400				+*		
500		+	+	-	+	+
+ indicates effect present, - indicates effect absent, blank cell indicates no data. * indicates indirect data from delay in balanopreputial separation data.						

Summary of Sulfoxaflor Leydig Cell Tumour MoA

The proposed MoA for sulfoxaflor-induced Fischer 344 rat Leydig cell tumour promotion is

through dopamine enhancement potentially mediated by agonism of the molecule on neuroendocrine dopaminergic nAChRs within the median eminence in the rat. The relevant end points for this MoA are summarised on table 6.5.4.4-13. This analysis is based on the mechanistic and standard, repeat-dose toxicity studies in rats administered sulfoxaflor. A summary of the data supporting each key event is presented below.

With respect to dose-response, the subtle nature of the effects observed ensured that no precursor key events were seen at 100ppm, only at 500ppm. A dose-response relationship for these apical end point effects existed with increased testis size and increased incidence of bilateral tumours at 500ppm. Due to the high background incidence of these tumours in Fischer rats, the lack of a response for precursor key events with the MoA analysis at the 100ppm dose level is not surprising.

Summary of Key Event #1: Increased Dopamine Release via nAChR Agonism

The release of dopamine via central nAChR agonism by sulfoxaflor has not been tested directly due to the lack of characterised nAChRs within the median eminence and inherent complexity of the biology of this system; however, because inhibition of prolactin release (Key Event #2) is primarily driven by dopamine release, the connection has been established with pharmaceutical dopamine agonists (*Prentice et al., 1992*). Central nicotinic acetylcholine receptors (nAChRs), such as α4β2 and α4α6β2 nAChRs, play a key regulatory role in dopamine release from non-neuroendocrine dopaminergic neurons in the brain (*Maskos, 2010*). Partial agonists to the α4β2 nAChR have been used as smoking cessation drugs (e.g., Tabex or cytisine) by causing release of dopamine in smaller portions to compensate for nicotine withdrawal (*Cassels et al., 2005*). The insecticidal mode-of-action for sulfoxaflor was binding to the insect nAChR, and it has been shown that sulfoxaflor was also an agonist to the rat foetal muscle subtype of the nAChR. It is plausible that the LCT promotion seen in the rat chronic/carcinogenicity study was through subtle, but prolonged, agonism at the central nAChRs within the median eminence causing release of dopamine and inhibition of prolactin release from the pituitary gland.

Summary of Key Event #2: Decreased Serum Prolactin Levels

In direct response to Key Event #1 of dopamine release from the hypothalamus to the anterior pituitary gland, prolactin secretion to the systemic circulation is inhibited. Levels of serum prolactin were measured in the LCT MoA study at 2-, 4-, and 8-weeks of exposure to 0, 25, 100, or 500ppm sulfoxaflor in Fischer rats. There was no effect of sulfoxaflor treatment on serum prolactin levels after two weeks of treatment; however, there was a 1.7-fold decrease in serum prolactin at 4 weeks in the 500 ppm group with a concomitant 2-fold increase in serum LH levels (see Key Event #5). The effect on prolactin levels was not observed at the 8-week time point, which suggests compensation by the HPG axis.

Summary of Key Event #3: Downregulation of LHR Gene Expression in Leydig Cells

Consistent with MoA #9 and the decreased Prl levels in the 4-week Fischer rat hormone data in Key Event #2, there was a ~1.6-fold dose-dependent decrease in LHR gene expression at the 4-week, but not 8-week, time point. In addition, there was a decrease in PrlR gene expression as well at the 4-week, but not 8-week, time point. While not a robust difference, the magnitude of gene expression changes is consistent with the dynamic range of these genes

in vivo and likely represents a biologically meaningful effect based on alterations in hormone levels. This conclusion is supported by a recent publication where administration of exogenous Prl to rats for 4-weeks resulted in a ~2-fold increase in LHR gene expression (*Williams et al., 2007*).

Summary of Key Event #4: Decreased Serum Testosterone Levels

Downregulation of the LHR in Key Event #3 leads to a transient decrease in serum testosterone levels in Key Event #4 (*Cook et al., 1999*). In LCT MoA experiments with the dopaminergic pharmaceutical agent mesulergine, serum testosterone levels were similar to controls at 2-weeks of treatment, slightly lower than controls at 4 weeks, returned to baseline by 10 weeks, and were elevated at 13 weeks (*Prentice et al., 1992*). Within the sulfoxaflor LCT MoA study, there were no decreases in serum testosterone levels at the 2-, 4-, or 8-week time point. However, in the two-generation reproductive toxicity study, there was a treatment-related delay in balanopreputial separation (BPS) for male offspring in the high-dose group of 400ppm sulfoxaflor. The process of BPS as a pubertal onset marker in a male rat is dependent on androgen levels, as testosterone injection to castrated rats is sufficient to induce BPS (*Korenbrot et al., 1977*). Therefore, in order for sulfoxaflor to induce a delay in BPS within the two-generation reproductive toxicity study, there had to be a decrease in testosterone levels during postnatal development. Further support to this statement is the fact that dopamine agonists such as bromocriptine induce a delay in male rat BPS (*Marty et al., 2001*).

Summary of Key Event #5: Increased Serum LH Levels

Common to most hormone-based LCT MoAs is an increase in serum LH acting as the causative agent for providing trophic stimulus of Leydig cells towards hyperplasia and eventually adenomas. The dopamine agonism/enhancement MoA is no exception to an eventual increase in LH leading to LCTs. With respect to sulfoxaflor, there was an increase in serum LH levels at the 4-week timepoint in Fischer rats, consistent with timing of decreased prolactin level, which was observed in the LCT MoA study.

Summary of Key Event #6: Promotion of Leydig Cell Tumourigenesis

In a rat chronic/carcinogenicity study, Fischer 344 rats were given 0, 25, 100, or 500ppm sulfoxaflor for 24 months. There was a treatment-related increase in testis weight at 100 and 500ppm that was due to an increased size of Leydig cell tumours (LCT) in these animals. Histopathological results confirmed that there was no increase in the overall incidence of LCT across the groups with 88, 92, 90, and 92% of male rats with these tumours at 0, 25, 100, and 500ppm, respectively. However, there was a significant increased incidence of animals with bilateral LCT at 500ppm, which is also attributed to increased promotion as the Leydig cell hyperplasia (mass smaller than one seminiferous tubule) grew to the size of a Leydig cell tumour (mass larger than one seminiferous tubule).

Before these findings were observed at the two-year time point of the rat chronic/carcinogenicity study, the only related effect was limited a slight 2.4-day delay in balanopreputial separation (BPS) at 400ppm in the two-generation study in Crl:CD(SD) rats. There were no other effects on other reproduction-related (i.e., androgen-mediated) end points, suggesting a subtle, transient alteration in testosterone levels. The end points that were within normal limits included:

- Testes, epididymides, accessory glands in F344 rats, CD rats or CD-1 mice (CD-1 mice dose levels 20× rat LOEL and 80× rat NOEL; Thomas et al., 2010)
- Sperm parameters (counts, motility, morphology)
- Reproduction – fertility, mating indices, time to mating
- Development, including in the developmental neurotoxicity study
- Markers of androgenic/anti-androgenic effects
- Male anogenital distance

E. Strength, consistency, and specificity of association of effects with key events:

The biological processes resulting in rat Leydig cell tumours have been reviewed extensively (*Cook et al., 1999; Clegg et al., 1997 and Prentice and Miekle 1995*). LCTs initially appear as hyperplasia of interstitial cells that can grow with age to the diameter of a single normal seminiferous tubule, at which point they are classified as adenomas per guidance from the National Toxicology Program (NTP) (*Boorman et al., 1990; Boorman et al., 1987*).

Results from the LCT MoA revealed a dose-dependent increase in LH concentrations concomitant with a dose-dependent decrease in Prl levels for Fischer rats at the 4-week time point. There was no effect of treatment on Prl, LH, or T at all other time points. Consistent with MoA #9, and the decreased Prl levels in the 4-week Fischer rat hormone data, was a dose-dependent decrease in LHR gene expression at the 4-week, but not 8-week, time point. While not statistically significant, the magnitude of gene expression changes is consistent with the dynamic range of these genes *in vivo* and likely represents a biologically meaningful effect based on alterations in hormone levels.

Consistency is difficult to ascertain when evaluating hormone data due to inherent variability, feedback compensation by the HPG axis, and the very long latency for the apical end point effect of Leydig cell hyperplasia and tumours. With respect to dose-response, due to the subtle nature of the effects no precursor key events were observed at 100ppm, but only at 500ppm. A dose-response relationship for these effects existed (i.e., 500ppm showed a greater effect across all key events than 100ppm); however, as sulfoxaflor merely increased the magnitude (i.e., size) of Leydig cell tumours due to the high background levels of these tumours in Fischer rats, the lack of a response at the lower 100ppm dose level may be masked and the result itself is not surprising.

The specificity of the data for MoA #9 is the decrease in circulating serum prolactin levels and decreased LHR gene expression. These findings would only be observed with MoA #9 and is not associated with the other eight possible MoAs leading to LCT (see Alternative MoA Analysis below). Furthermore, the decrease in serum Prl was associated with a compensatory increase in serum LH, which in turn could act as the primary trophic stimulus over a two-year Fischer rat oncogenicity study leading to LCT promotion. Due to the persistent compensatory nature of the hypothalamic-pituitary-gonadal (HPG) axis, coupled with the fact that chronic (i.e., two-years) sulfoxaflor exposure was required for increased LCT size in Fischer rats, it is not surprising that the changes observed in the hormone data are temporal in nature. In fact, conclusive Leydig cell hyperplastic effects in the guideline toxicity studies occurred only at the two-year time point.

F. Biological plausibility and coherence:

Dietary administration of sulfoxaflor to Fischer rats results in the early key events (decrease in serum prolactin and LHR gene expression) that lead to an increase in serum LH levels. The MoA demonstrated for sulfoxaflor is consistent with well-known MoA for dopamine agonists/enhancers and is consistent with current understanding of hormone-based Leydig cell tumourigenesis. The data for sulfoxaflor are entirely consistent with a non-genotoxic, threshold, MoA.

G. Assessment of postulated Sulfoxaflor Fischer rat Leydig cell tumour MoA:

The data for sulfoxaflor support a subtle, but chronic, enhancement of dopamine release, and subsequent inhibition of prolactin release from the pituitary gland, ultimately leading to a dopamine agonism/enhancement LCT MoA in a uniquely susceptible animal model, the Fischer 344 rat. The MoA demonstrated for sulfoxaflor is consistent with the literature and with current understanding of rodent Leydig cell tumours. As mentioned previously, the data for sulfoxaflor are consistent with this non-genotoxic MoA of the Leydig cell. *In vitro* and *in vivo* studies show sulfoxaflor does not have a genotoxic MoA (see below).

The data for sulfoxaflor are judged with a moderate degree of confidence to adequately explain the increase in size of Fischer rat Leydig cell tumours following chronic dietary administration of sulfoxaflor, and judged with a very high degree of confidence to support a hormonally-mediated, threshold based, nonlinear MoA.

The sulfoxaflor MoA analysis is summarised in Table 6.5.4.4-14 in terms of the criteria for the human relevance framework.

H. Consideration of alternative mode of actions:

In the process of conducting and evaluating experiments aimed at testing the proposed MoA for sulfoxaflor promotion of Leydig cell tumours in Fischer 344 rats, it was possible to rule out a number of alternative MoAs. Each of these alternative MoAs will be considered in turn and direct and/or indirect data generated with sulfoxaflor will be discussed. Wherever possible, sulfoxaflor will be compared to prototypical compounds which are known to cause LCT or LC hyperplasia through these alternative MoAs.

Table 4.10.3.1.Study 10.14 (DAR Table 6.5.4.4-14.) Analysis of sulfoxaflor Rodent Leydig Cell Tumour MoA	
Key Event #1: Increased Dopamine Release via nAChR Agonism Key Event #2: Decreased Serum Prolactin Levels Key Event #3: Downregulation of LHR Gene Expression Key Event #4: Decreased Serum Testosterone Levels Key Event #5: Increased Serum LH Levels Key Event #6: Promotion of Leydig Cell Tumourigenesis	
Strength of association	+ Moderate
Consistency of association	+ Moderate
Specificity of association	+ Moderate
Dose-response concordance	+ Moderate
Temporal relationship	+/- Weak
Coherence & plausibility	+ Plausible; + Coherence

1) Mutagenicity – Not Plausible

Mutagenic agents either initiate Leydid cells and then LH would promote the development of the tumour, or they act via an unidentified hormonal mechanism (that may or may not be related to their mutagenic or clastogenic activity). An example of a mutagenic compound that causes LCTs is cadmium.

Sulfoxaflor was clearly negative in the battery of *in vitro* and *in vivo* genotoxicity assays for mutagenicity and clastogenicity. These included the bacterial reverse mutation (Ames) test, *in vitro* mammalian chromosome aberration (RLCAT) test, the *in vitro* mammalian cell gene mutation (CHO/HGPRT) test, and the mammalian erythrocyte micronucleus (MNT) test. The design and results of these studies are summarised below in Table 6.5.4.4-15.

In addition, if the Leydig cell effects in F344 rats were caused by a genotoxicity MoA, it would be expected to have an earlier onset. From the two-year rat study, when considering Leydig cell tumour incidence in rats from all treatment groups that were moribund or found dead prior to test day 500, there was no evidence of earlier onset of LCT.

Based on the weight-of-evidence, considering both direct data which shows sulfoxaflor is non-genotoxic, and indirect data generated from the toxicology package that indicates no earlier onset of Leydig cell tumours, a mutagenicity MoA is not a plausible alternative MoA for the Leydig cell effects seen in F344 rats after two years of treatment with sulfoxaflor.

Table 4.10.3.1.Study 10.15 (DAR Table 6.5.4.4-15): Summary of Evidence for Non-Genotoxicity of Sulfoxaflor				
Test	**Test System**	**Study design**	**Result**	**Report ref. (Study ID)**
In vitro genotoxicity tests				
Bacterial Reverse Mutation Test	*S.typhimurium* TA 98, TA 100, TA 1535 & TA 1537 *E. coli*, WP2*uvr*A	33.3, 100, 333, 1000, 2500, and 5000µg per plate +/- S9	**Negative**	Mecchi, 2007 (071110)
Mammalian Chromosome Aberration Test	Rat lymphocytes	4 hr treatment: 0, 693.3, 1386.5, and 2773µg/ml +/- S9; 24 hr treatment: 0, 173.3, 346.6, and 693.3µg/ml	**Negative**	Schisler *et al.*, 2007a (071029)
Mammalian Cell Gene Mutation Test	Chinese hamster ovary cells CHO/HGPRT	0, 173.3, 346.6, 693.3, 1386.5, and 2773µg/ml +/- S9	**Negative**	Schisler *et al.*, 2007b (071030)
In vivo genotoxicity tests – Somatic cells				
Mammalian Erythrocyte Micronucleus Test	Mouse bone marrow polychromatic erythrocytes	Male and female CD-1 mice, 6/sex/dose, single oral gavage on two consecutive days at 0, 100, 200, and 400 mg/kg/day	**Negative**	LeBaron and Schisler, 2009 (071100)

2) Androgen receptor antagonism – Not Plausible

Androgen receptor antagonists compete with testosterone and DHT for binding to the androgen receptor. This competition reduces the androgenic signal to the hypothalamus and adenohypophysis, resulting in an increase in LH secretion with a concomitant elevation of testosterone secretion, resulting in the development of LCTs. Direct data obtained with sulfoxaflor shows that, although there was an indication that it is a potential binder (table 6.5.4.4-16) to a fragment (i.e., ligand binding domain) of the AR in a non-cell-based binding assay, there was no effect on agonism or antagonism within the AR transactivation assay (figure 6.5.4.4-6: positive control agonism; figure 6.5.4.4-7: positive control antagonism; figure 6.5.4.4-8: sulfoxaflor). Based on this, speculative sulfoxaflor-related denaturation of the androgen receptor or other non-specific interaction cannot be ruled out as a potential mechanism for the response observed in the AR-binding assay, as no biological effect was identified in an AR-mediated transactivation assay. Therefore, there appears to be no biological relevance of the potential AR binding result.

Table 4.10.3.1.Study 10.16 (DAR Table 6.5.4.4-16): Relative binding affinity of sulfoxaflor in AR fluorescent polarization assay			
AR Binding	**IC50 Mean (M)**	**IC50 Std Error**	**Relative Binding Affinity Mean**
DHT	6.37E-09	1.25E-09	NA
XDE-208	4.96E-04	1.76E-04	1.37E-03

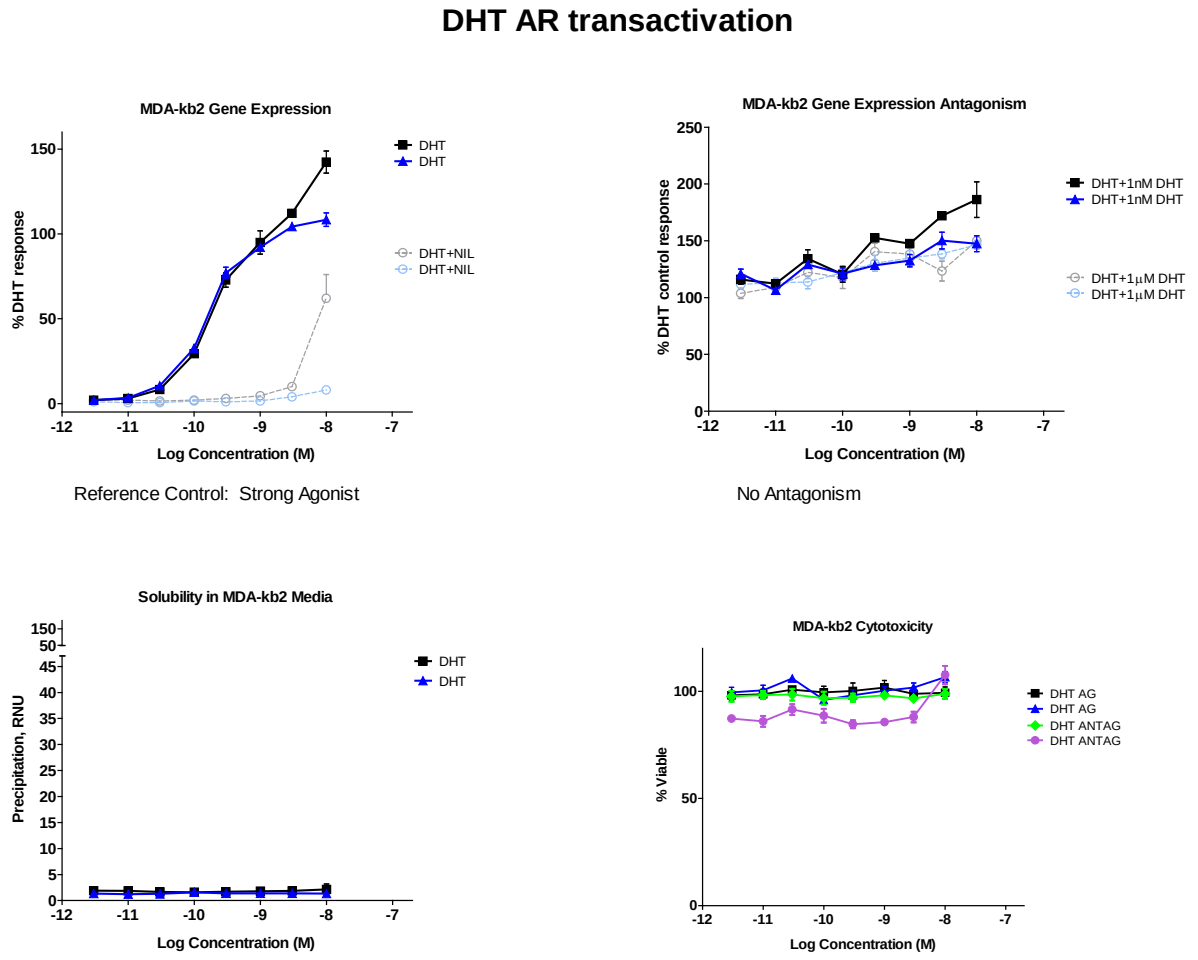


Figure 4.10.3.1.Study 10.6 Figure 6.5.4.4-6. Reference Agonist Control, DHT – AR Transcriptional Activation (agonism and antagonism)

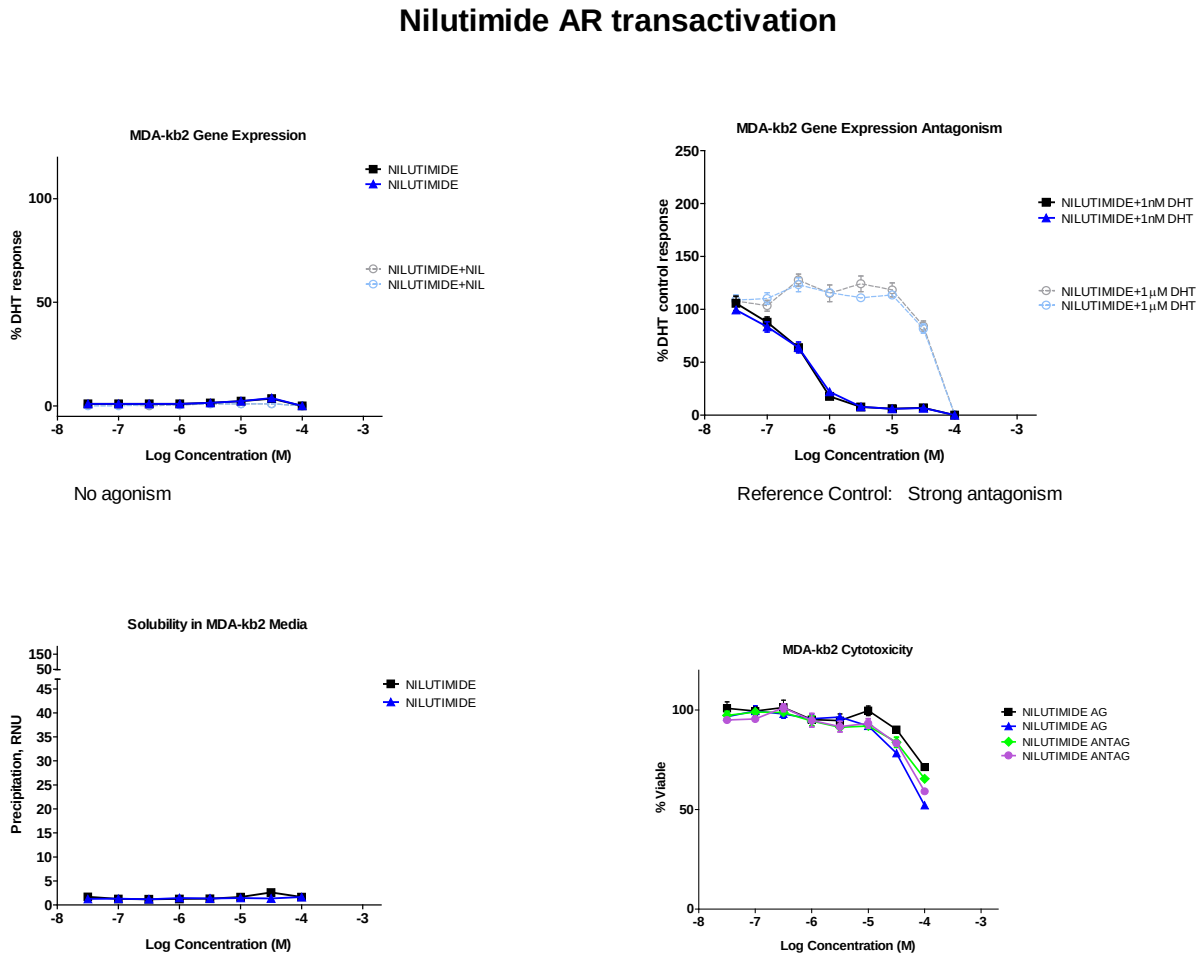


Figure 4.10.3.1.Study 10.7 (DAR Figure 6.5.4.4-7): Reference Antagonist Control, Nilutamide – AR Transcriptional Activation (agonism and antagonism)

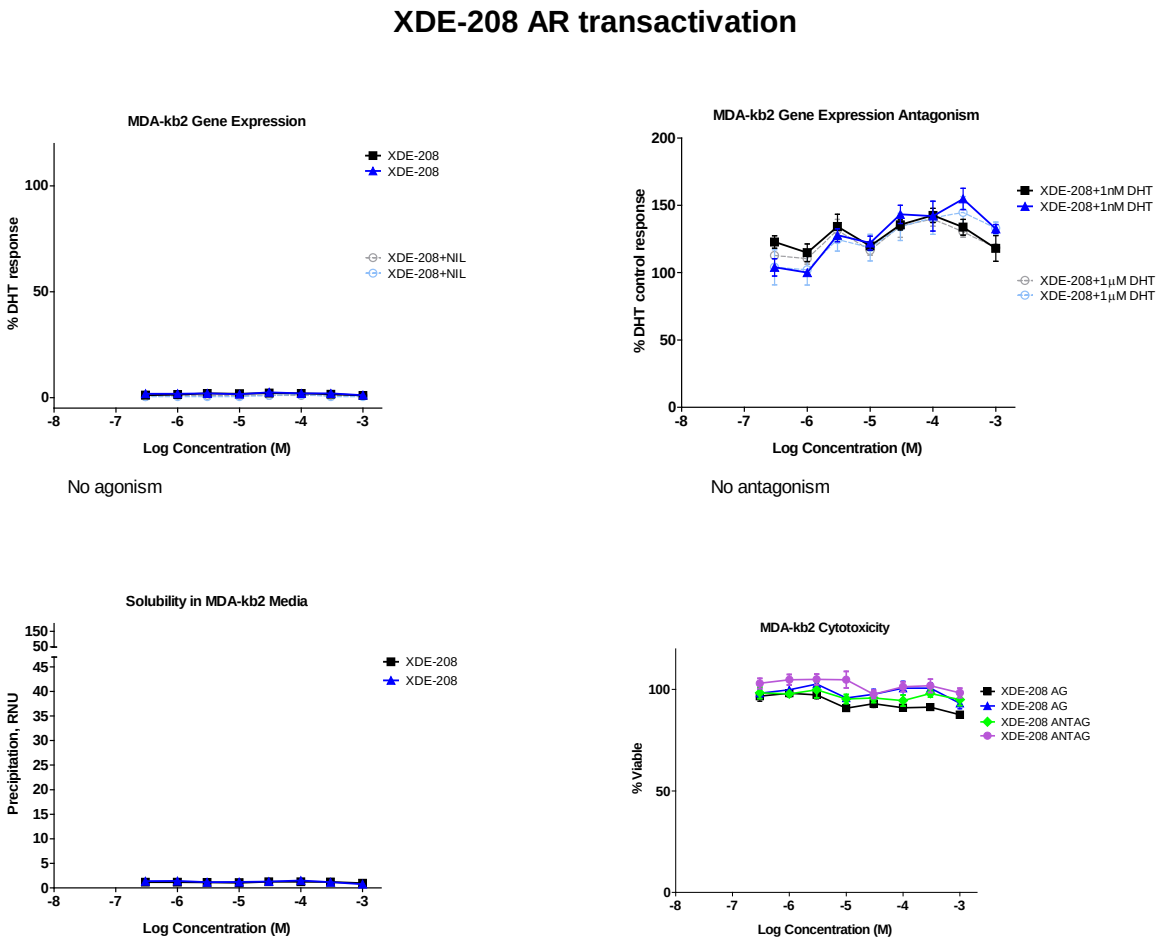


Figure 4.10.3.1.Study 10.8 (DAR Figure 6.5.4.4-8): Sulfoxaflor – AR Transcriptional Activation (agonism and antagonism).

The lack of an increase above historical control background of LCTs at 1 year of treatment with sulfoxaflor is in contrast to the prototypical AR antagonists such as vinclozolin and flutamide. Vinclozolin and flutamide also have a fingerprint of effect that includes reduced anogenital distance, male reproductive malformation (such as hypospadias) and reduced accessory sex gland weights in reproductive toxicity studies. Sulfoxaflor did not cause any consistent androgen-associated effects in the toxicology package that would indicate an AR antagonist MoA. The study most sensitive to these types of end points is the two generation reproductive toxicity study. This study showed no treatment-related effects on anogenital distance, no effects on testis or accessory sex gland (i.e., prostate, seminal vesicle, and epididymis) weight or histopathology, no evidence of malformations (e.g., hypospadias or ectopic testes), and no effects on mating, fertility, time to mating, or gestation length.

Table 4.10.3.1.Study 10.17 (DAR Table 6.5.4.4-17): Summary of Evidence for Absence of AR Antagonism of XDE-208

Test	Test System	Study design	Result	Report ref. (Study ID)
***In vitro* tests:**				
Androgen Receptor Binding Assay	Androgen receptor ligand binding domain	4-8 hr treatment: -7.52 to -3 logM	Weak Positive	Toole, 2011
Androgen Transactivation Assay	MDA-kb2 human breast carcinoma cell line with luciferase reporter gene for the androgen response element (ARE)	24 hrs treatment: -6.52 to -3.0 logM	Negative	Toole, 2011
***In vivo* tests:**				
Two-generation reproduction study	Rat/CD	0, 25, 100, 400 ppm for ~ 10 weeks prior to breeding, through breeding, gestation and lactation for 2 generations M: 0, 1.5-1.7, 6.1-6.9, 24.6-28.1 (range of doses for F1 ad F2 males) F: 0, 1.6-2.1, 6.6-8.4, 26.8-34.0 mkd (range of doses for F1 and F2 females)	Slight delay in BPS in 400 ppm F1 males but no other indicators of androgenic or anti-androgenic effects	Rasoulpour *et al*., 2010b (091023)

* BPS, Balanopreputial separation

Based on the weight-of-evidence, considering both direct data which shows sulfoxaflor is negative for AR transactivation for agonism and antagonism, and indirect data generated from the toxicology package that indicates no AR antagonist MoA (table 6.5.4.4-17), an AR antagonist MoA is not a plausible alternative MoA for the Leydig cell effects seen in F344 rats after two years of treatment with sulfoxaflor.

3) Oestrogen receptor agonism/antagonism – Not Plausible

Oestrogen receptor agonists/antagonists result in changes in oestradiol levels which ultimately cause an increase in LH levels resulting in the development of LCTs. Direct data obtained with sulfoxaflor show that it is negative for ER binding and transactivation (agonism and antagonism), even when tested up to very high concentrations *in vitro* (figure 6.5.4.4-9: positive control agonism; figure 6.5.4.4-10: positive control antagonism; figure 6.5.4.4-11: sulfoxaflor).

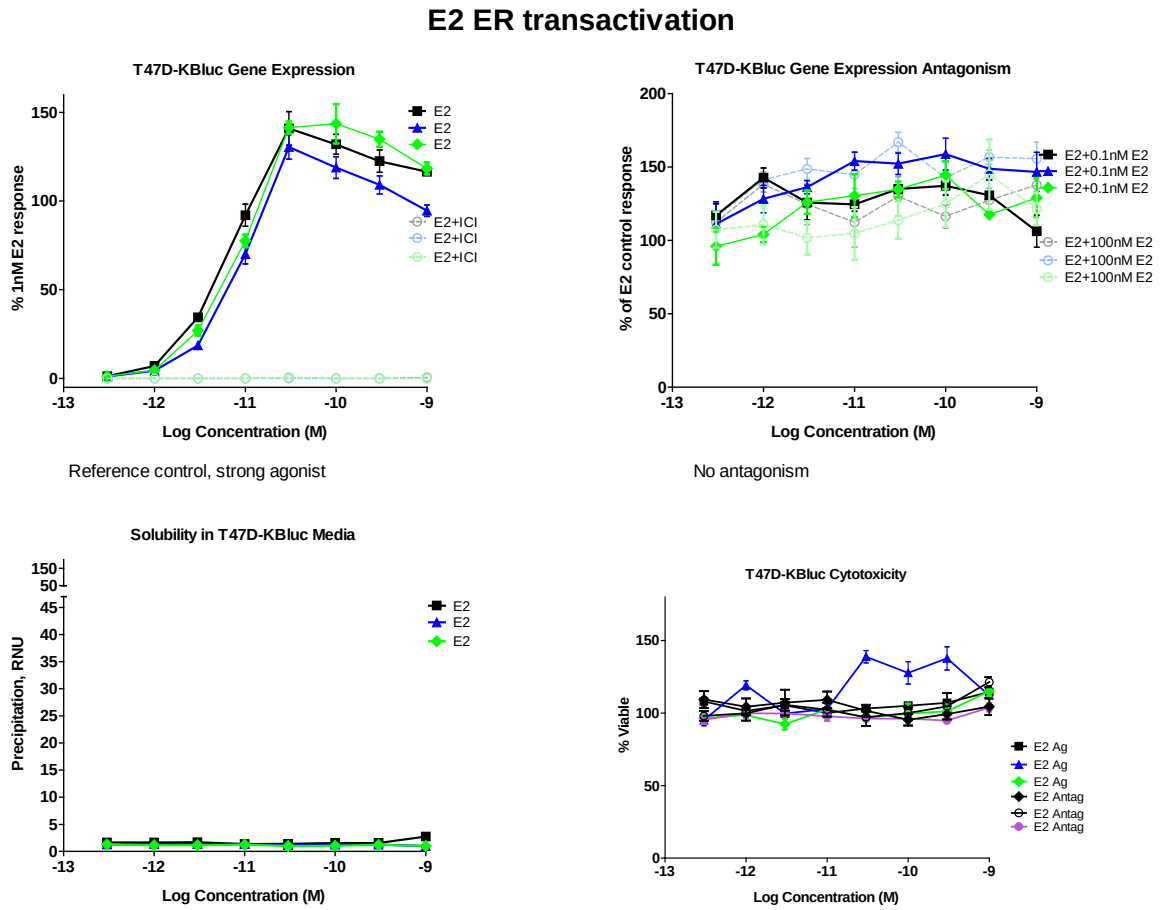


Figure 4.10.3.1.Study 10.9 (DAR Figure 6.5.4.2/3-9): Reference Control E2 – ER Transcriptional Activation (agonism and antagonism).

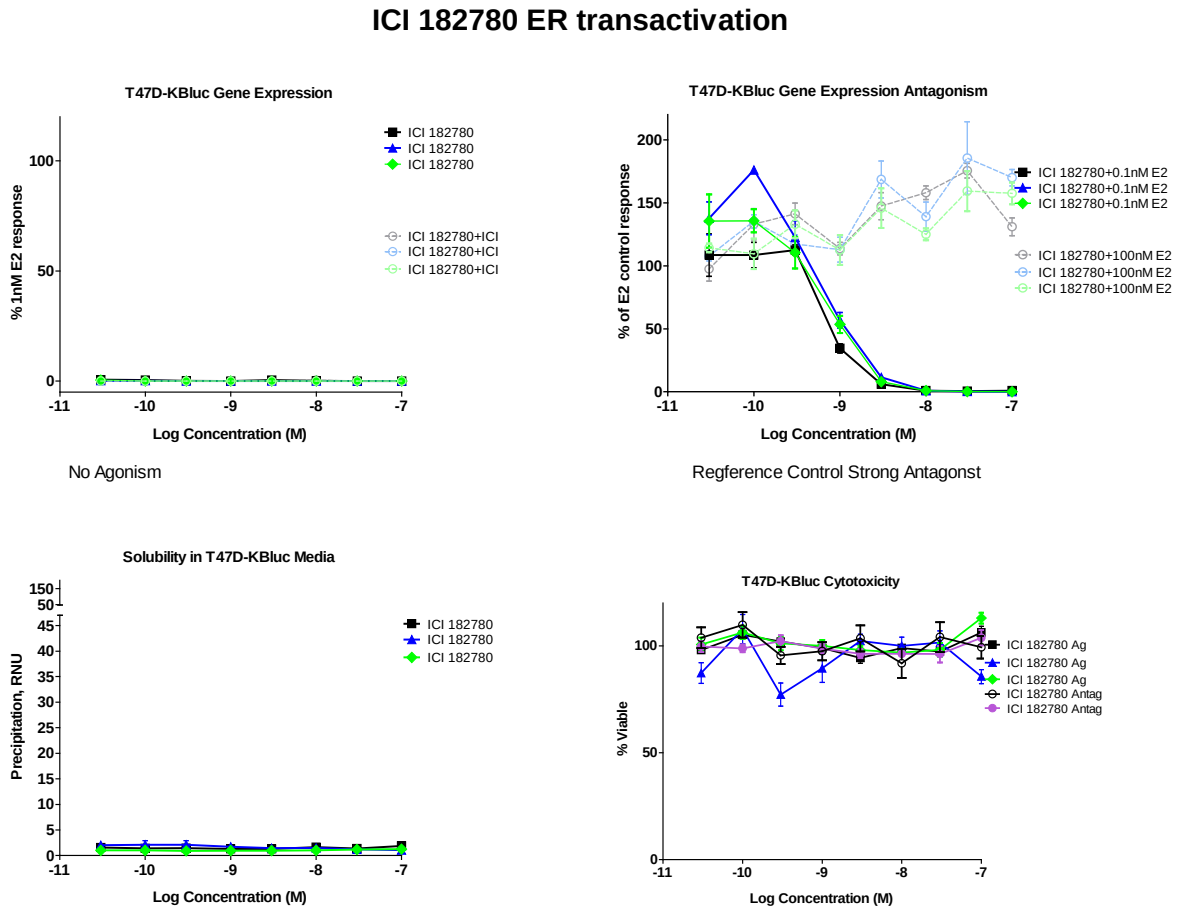


Figure 4.10.3.1.Study 10.10 (DAR Figure 6.5.4.4-10): Reference Control ICI 182780 – ER Transcriptional Activation (agonism and antagonism).

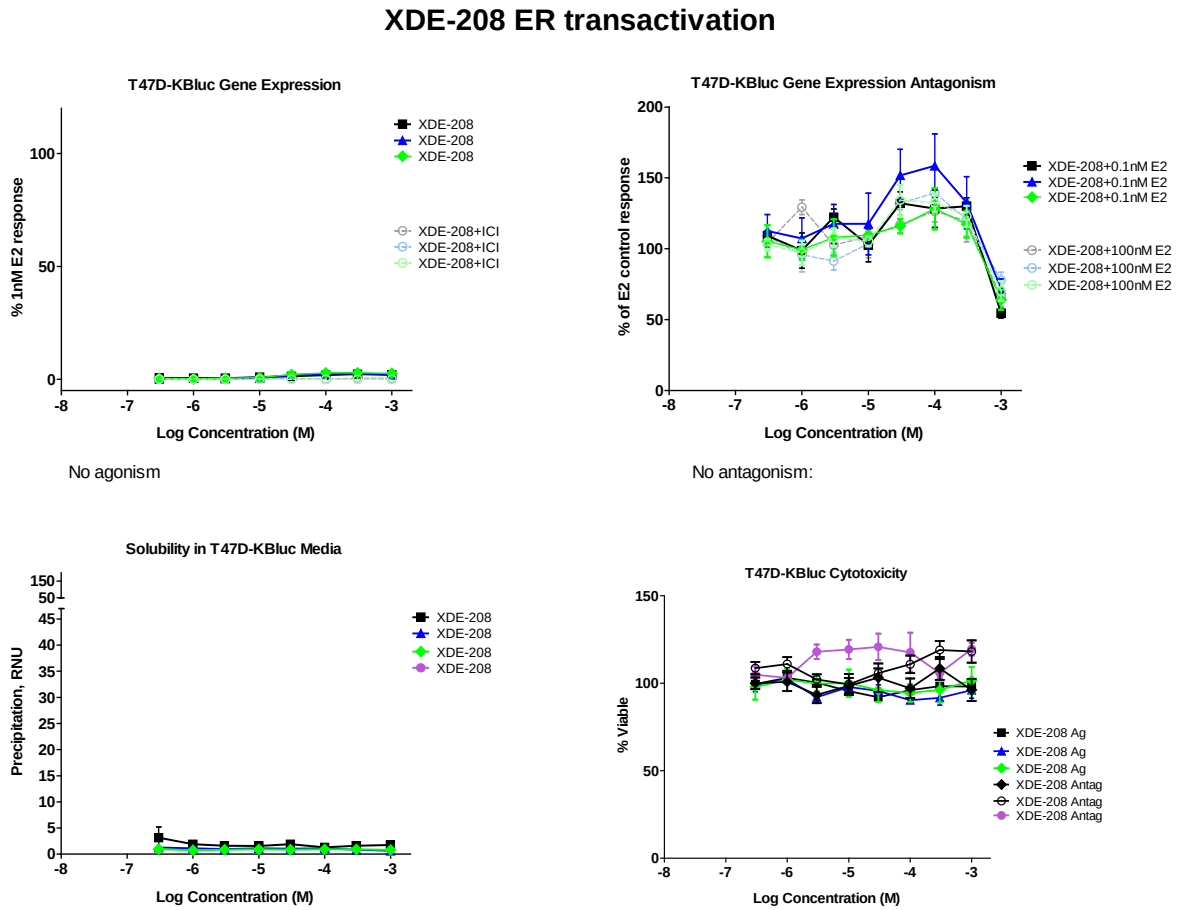


Table 4.10.3.1.Study 10.11 (DAR Figure 6.5.4.2/3-11): Sulfoxaflor – ER Transcriptional Activation (agonism and antagonism).

Interestingly these types of compounds induce LCTs almost exclusively in the mouse rather than the rat. Sulfoxaflor does not induce LCTs in the mouse: despite the fact that dose levels in the mouse oncogenicity study were more than 4× times higher than in the rat oncogenicity study, there were no effects on reproductive organs, including the testes, in that study. Prototypical oestrogen receptor agonist/antagonists, such as diethylstilbestrol, cause effects on vaginal patency, oestrus cyclicity, female reproductive tract histopathological and organ weight effects. There were no effects on female reproductive indices, organ weights, reproductive histopathology, vaginal patency, or oestrus cyclicity in any sulfoxaflor rodent study including the two-generation reproductive toxicity study.

Table 4.10.3.1.Study 10.18 (DAR Table 6.5.4.4-18): Summary of Evidence for Absence of ER Antagonism by Sulfoxaflor				
Test	**Test System**	**Study design**	**Result**	**Report ref. (Study ID)**
***In vitro* tests:**				
Oestrogen Receptor Binding Assay	Oestrogen receptor alpha	2 hr treatment: -7.52 to -3 logM	Negative	Toole (2011)
Oestrogen Transactivation Assay	T47D-Kbluc human breast carcinoma cell line with luciferase reporter gene for the oestrogen response element (ARE)	24 hrs treatment: -6.52 to -3.0 logM	Negative	Toole (2011)
***In vivo* tests:**				
Two-generation reproduction study	Rat/CD	0, 25, 100, 400 ppm 208 for ~ 10 weeks prior to breeding, through breeding, gestation and lactation for 2 generations M: 0, 1.5-1.7, 6.1-6.9, 24.6-28.1 (range of doses for F1 ad F2 males) F: 0, 1.6-2.1, 6.6-8.4, 26.8-34.0 mkd (range of doses for F1 and F2 females over course of treatment)	No indicators of oestrogenic or anti-oestrogenic effects: no treatment-related effects on female reproductive organ weights, reproductive histopathology, vaginal patency, estrus cyclicity, mating, fertility, time to mating, or gestation length	Rasoulpour *et al*., 2010b (091023)

Oncogenicity study	Mouse/CD1	M: 0, 25, 100, 750 ppm (0, 2.54, 10.4, 79.6 mkd) F: 0, 25, 250, 1250 ppm (0, 3.43, 33.9, 176 mkd)	No effect on testes or increase in incidence of Leydig cell tumours at dose levels 20× rat LOEL and 80× rat NOEL	Thomas *et al.* 2010 (081102)

Based on the weight-of-evidence, considering both direct data which shows sulfoxaflor is negative for ER binding and ER transactivation for agonism and antagonism, and indirect data generated from the toxicology package that indicates no ER antagonist MoA (table 6.5.4.4-18), an ER agonist/antagonist MoA is not a plausible alternative MoA for the Leydig cell effects seen in F344 rats after two years of treatment with sulfoxaflor.

4) 5-alpha reductase inhibition – Not Plausible

5-alpha reductase inhibitors result in decreased conversion of testosterone to dihydrotestosterone (DHT). This reduces the net androgenic signal received by the hypothalamus and pituitary, thereby causing a compensatory increase in LH levels, resulting in the development of LCT. The prostate is differentially sensitive to effects on DHT: for example, DHT has 5-fold greater affinity for AR than T (testosterone). Because of this, the prostate would be the most sensitive organ affected compared to other accessory sex glands. 5-alpha inhibitors can reduce prostate weight 20-30% although T can remain normal. There was no effect of sulfoxaflor on prostate weight in any *in vivo* study.

Interestingly, 5α-reductase inhibitors induce LCTs in mice and LC hyperplasia in rats. Sulfoxaflor does not induce LCTs in the mouse: despite the fact that dose levels in the mouse oncogenicity study were more than 4× times higher than in the rat oncogenicity study, there were no effects on reproductive organs, including the testes, in that study. In addition, in the Leydig cell tumour MoA study where F344 and Crl:CD(SD) rats were treated with 0, 25, 100 or 500ppm sulfoxaflor, at 4 and 8 weeks there was no effect on 5-alpha-reductase (SDR5a1) gene expression levels in the testes of treated rats (figures 6.5.4.4-12 and figure 6.5.4.4-13).

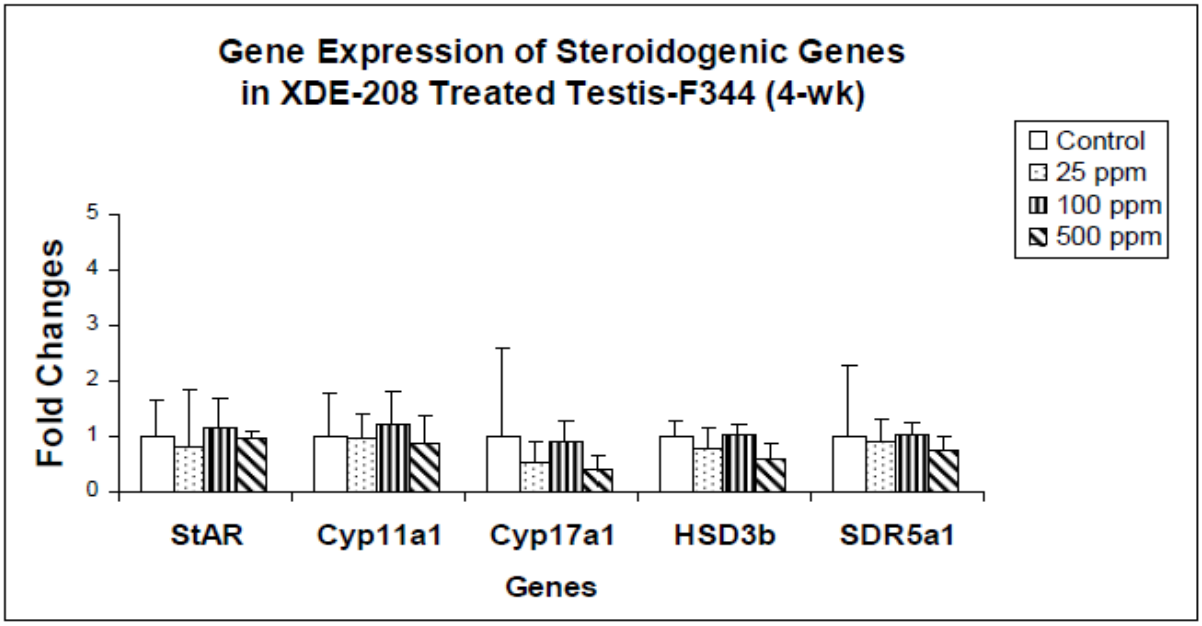


Table 4.10.3.1.Study 10.19 (DAR Figure 6.5.4.4-12): Gene expression of steroidogenic genes in sulfoxaflor treated testis in F344 rats (4-week treatment).

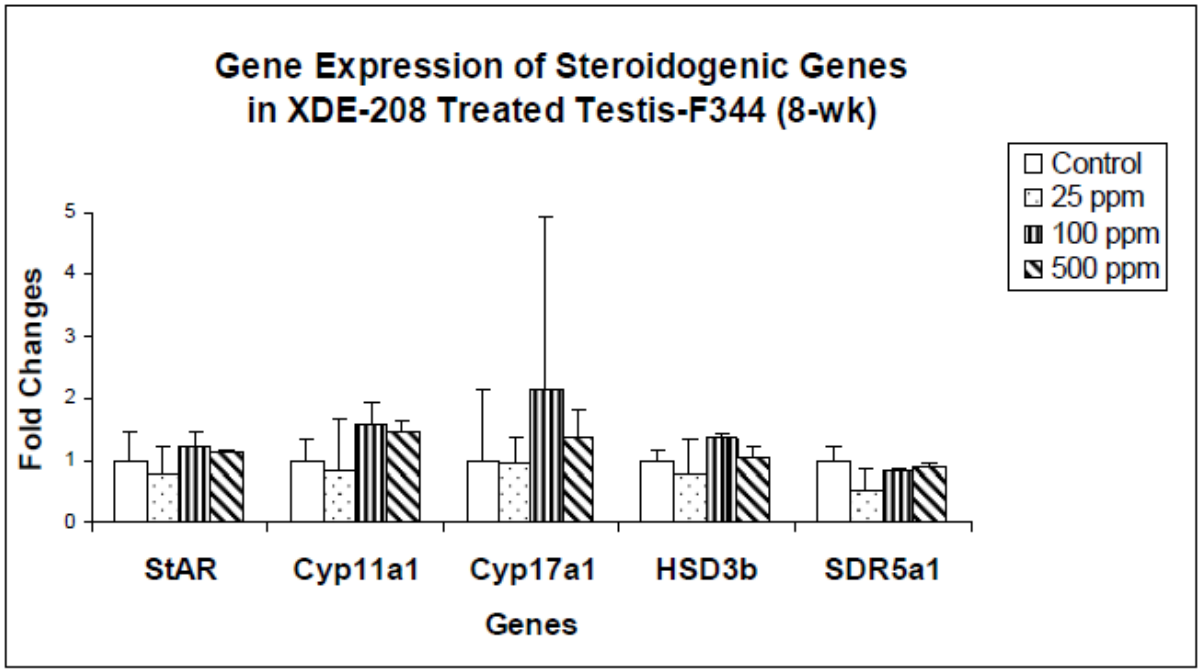


Figure 4.10.3.1.Study 10.13 (DAR Figure 6.5.4.4-13): Gene expression of steroidogenic genes in sulfoxaflor-treated testis in F344 Rats (8-week treatment).

The prototypical 5-alpha-reductase inhibitor is finesteride, which causes reduced anogenital distance, hypospadias and reductions in accessory sex gland organ weights. There was no indication of reduced anogenital distance or effects on reproductive organ weights in the two-generation reproductive toxicity study.

Based on the weight-of-evidence, considering both direct data which shows sulfoxaflor has no effect on testes 5-alpha reductase gene expression, and indirect data generated from the toxicology package that indicates no prostate effect (table 6.5.4.4-19), a 5-alpha reductase inhibition MoA is not a plausible alternative MoA for the Leydig cell effects seen in F344 rats after two years of treatment with sulfoxaflor.

5) Aromatase inhibition – Not Plausible

Inhibition of aromatase would result in decreased conversion of androstenedione to oestrone, and testosterone to oestradiol. This would result in an increase in LH levels leading to the development of LCTs. Direct data obtained with sulfoxaflor show that it is negative for aromatase inhibition when tested up to very high (i.e., super physiological) concentrations *in vitro* (figure 6.5.4.4-14, ASDN = 4-OH-androstenedione).

Table 4.10.3.1.Study 10.19 (DAR Table 6.5.4.4-19): Summary of evidence for absence of 5α-reductase inhibition by sulfoxaflor.

Test	Test System	Study design	Result	Report ref. (Study ID)
In vivo tests				

Leydig cell tumour MoA study	F344 and Crl:CD(SD) rats	0, 25, 100 or 500 ppm for up to 8 wks	No effect on SDR5a1 (5α-reductase) gene expression in the testes	Rasoulpour *et al.*, 2010a
Two-generation reproduction study	Rat/CD	0, 25, 100, 400 ppm 208 for ~ 10 weeks prior to breeding, through breeding, gestation and lactation for 2 generations M: 0, 1.5-1.7, 6.1-6.9, 24.6-28.1 (range of doses for F1 ad F2 males) F: 0, 1.6-2.1, 6.6-8.4, 26.8-34.0 mkd (range of doses for F1 and F2 females over course of treatment)	No treatment-related effects on anogenital distance, no effects on testis or accessory sex gland (i.e., prostate, seminal vesicle, and epididymis) weight or histopathology, no evidence of malformations like hypospadias or ectopic testes	Rasoulpour *et al.*, 2010b (091023)
Oncogenicity study	Mouse/CD1	M: 0, 25, 100, 750 ppm (0, 2.54, 10.4, 79.6 mkd) F: 0, 25, 250, 1250 ppm (0, 3.43, 33.9, 176 mkd)	No effect on testes or increase in incidence of Leydig cell tumours at dose levels 20X rat LOEL and 80X rat NOEL	Thomas *et al.* 2010 (081102)

Aromatase inhibitors, such as anastrozole, cause effects on mating and fertility indices as well as female reproductive organ weights and histopathology. There were no effects on mating, sperm parameters (counts, motility, morphology) or fertility indices in the two generation reproductive toxicity study with Sulfoxaflor.

Based on the weight-of-evidence, considering both direct data which shows sulfoxaflor has no effect aromatase activity, and indirect data generated from the two-generation reproduction study (table 6.5.4.4-20), an aromatase inhibition MoA is not a plausible alternative MoA for the Leydig cell effects seen in F344 rats after two years of treatment with sulfoxaflor.

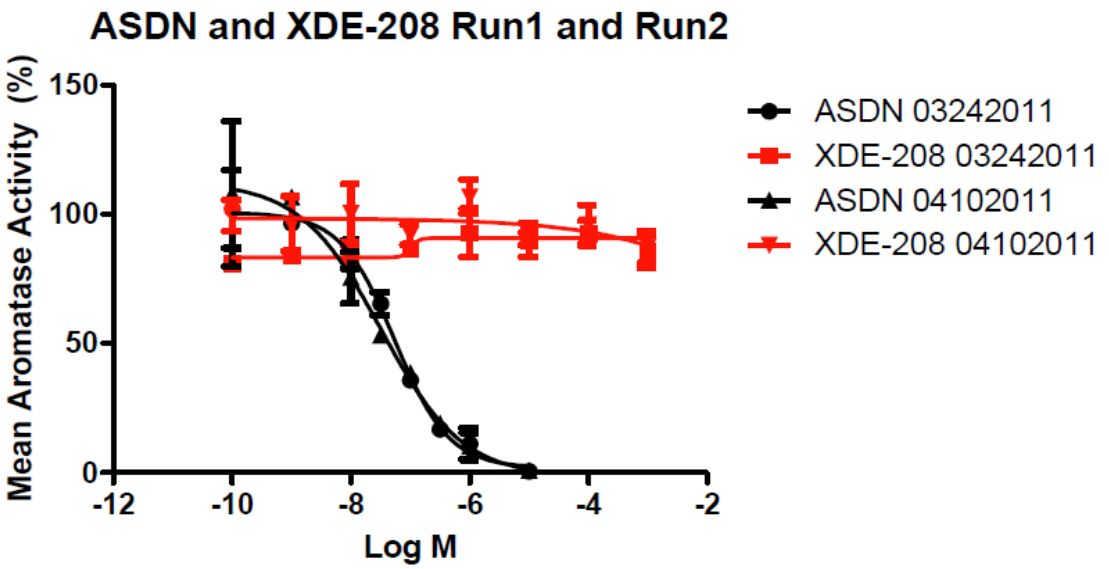


Figure 4.10.3.1.Study 10.14 (DAR Figure 6.5.4.4-14): Results of an Aromatase Inhibition Assay with sulfoxaflor.

6) Reduced testosterone biosynthesis – Not Plausible

Inhibition of testosterone biosynthesis would result in lower testosterone and oestradiol levels, and increased LH levels, resulting in the development of LCTs. Direct data is provided for sulfoxaflor from the Leydig cell tumour MoA study where F344 and Crl:CD(SD) rats were treated with 0, 25, 100 or 500ppm sulfoxaflor for up to 8 weeks. Gene expression analysis of testes mRNA from this study was conducted on a suite of steroidogenic enzymes to evaluate this potential alternate MoA. There was no dose-dependent effect of treatment on any measured gene in the steroidogenic pathway including *StAR* (steroidogenic acute regulatory protein), *Cyp11a1* (P450side chain cleavage), *Cyp17a1* (17α-hydroxylase), *HSD3b* (3-beta hydroxysteroid dehydrogenase), or *SDR5a1* (5-α reductase). If reduced testosterone biosynthesis were the operant MoA, one or more of these genes would be affected. Furthermore, the hormone panel data would have shown a sustained decrease in circulating levels of testosterone, which was not observed in the LCT MoA study (*Rasoulpour et al., 2010a*). Taken together these data, as well as a lack of female reproductive effects, refute decreased steroidogenesis (MoA #6) as the operant MoA.

Examples of testosterone biosynthesis inhibitors include lansoprazole and calcium channel blockers, which lead to effects on mating and fertility indices as well as reproductive organ weight and histopathology. While there was an increase in serum cholesterol (the starting material for steroidogenesis) associated with sulfoxaflor administration and a slight delay in preputial separation, there was no effect on female reproductive parameters, which would have been expected with this MoA as androgens are the precursors to oestrogens. No effects on mating, fertility, or reproductive organs were observed in the two-generation reproductive toxicity study.

Table 4.10.3.1.Study 10.20 (DAR Table 6.5.4.4-20): Summary of evidence for absence of aromatase inhibition of sulfoxaflor.				
Test	**Test System**	**Study design**	**Result**	**Report ref. (Study ID)**
***In vitro* tests**				
Aromatase Inhibition Assay	Human recombinant microsomes (Human CYP19 [Aromatase] and P450 reductase Supersomes™, Gentest™)	15 min treatment: -10.0 to -3.0 logM	Negative	Toole (2011)
***In vivo* tests**				

Two-generation reproduction study	Rat/CD	0, 25, 100, 400 ppm 208 for ~ 10 weeks prior to breeding, through breeding, gestation and lactation for 2 generations M: 0, 1.5-1.7, 6.1-6.9, 24.6-28.1 (range of doses for F1 ad F2 males) F: 0, 1.6-2.1, 6.6-8.4, 26.8-34.0 mkd (range of doses for F1 and F2 females over course of treatment)	No treatment-related effects on female reproductive organs weight or histopathology, no effects on mating, sperm parameters (counts, motility, morphology)fertility, time to mating, or gestation length	Rasoulpour *et al*., 2010b (091023)

Based on the weight-of-evidence (table 6.5.4.4-21), considering both direct data which shows sulfoxaflor has no effect on gene expression involved in the steroidogenic pathway and that there is no transient decrease in circulating levels of testosterone, and indirect data generated from the 2-generation reproduction study, a reduced testosterone synthesis MoA is not a plausible alternative MoA for the Leydig cell effects seen in F344 rats after two years of treatment with sulfoxaflor.

Table 4.10.3.1.Study 10.21 (DAR Table 6.5.4.4-21). Summary of evidence for absence of inhibition of testosterone biosynthesis by sulfoxaflor.				
Test	**Test System**	**Study design**	**Result**	**Report ref. (Study ID)**
***In vivo* tests**				
Leydig cell tumour MoA study	F344 and Crl:CD(SD) rats	0, 25, 100 or 500 ppm for up to 8 wks	No effect on StAR (steroidogenic acute regulatory protein), Cyp11a1 (P450side chain cleavage), Cyp17a1 (17alpha-hydroxylase), HSD3b (3-beta hydroxysteroid dehydrogenase), or SDR5a1 (5α-reductase) gene expression in the testes No ↓T at any timepoint (2, 4, 8 wks)	Rasoulpour *et al.*, 2010a
Two-generation reproduction study	Rat/CD	0, 25, 100, 400 ppm 208 for ~ 10 weeks prior to breeding, through breeding, gestation and lactation for 2 generations M: 0, 1.5-1.7, 6.1-6.9, 24.6-28.1 (range of doses for F1 ad F2 males) F: 0, 1.6-2.1, 6.6-8.4, 26.8-34.0 mkd (range of doses for F1 and F2 females over course of treatment)	No indicators of estrogenic or anti-estrogenic effects: no treatment-related effects on female reproductive organ weights, reproductive histopathology, vaginal patency, estrus cyclicity, mating, fertility, time to mating, or gestation length	Rasoulpour *et al*., 2010b (091023)

7) Increased testosterone biliary elimination – Not Plausible

Increased biliary elimination of testosterone would cause lower testosterone levels, and increased LH levels, resulting in the development of LCTs. Based on known nuclear receptor-mediated liver effects with sulfoxaflor administration, this MoA was assessed and direct data is provided for sulfoxaflor from the Leydig cell tumour MoA study. Support for MoA #7 would be visualised by a dose-dependent increase in the amount of T-derived radioactivity eliminated in the bile. However, there were no statistically significant (alpha =

0.05) or treatment-related differences in the mean ^{14}C-testosterone-derived radioactivity excreted in the bile across all dose groups, per time intervals, for F344/DuCrl rats (figure 6.5.4.4-15). Bile flow was very similar for the respective dose groups and time intervals (figure 6.5.4.4-16). Similarly, there was no difference in plasma testosterone levels with treatment nor were there any changes in the plasma timecourse for elimination of testosterone from the blood (figure 6.5.4.4-17). These data clearly refute MoA #7 (biliary elimination of testosterone) as the operant MoA.

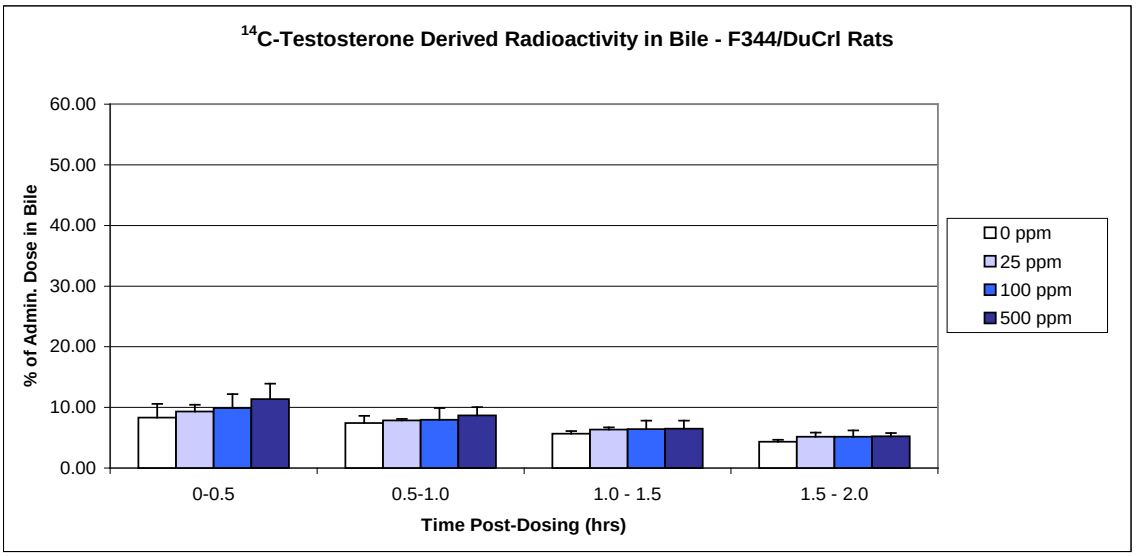


Figure 4.10.3.1.Study 10.15 (DAR Figure 6.5.4.4-15): ^{14}C-Testosterone derived Radioactivity in Bile – F344/DuCrl and Crl:CD(SD) Rats.

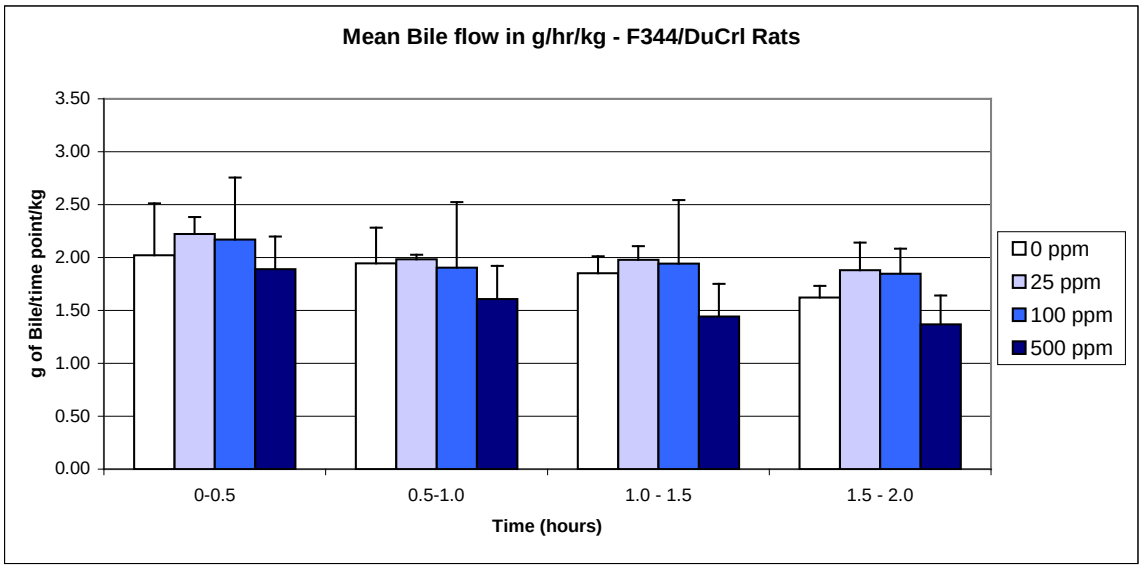


Figure 4.10.3.1.Study 10.16 (DAR Figure 5.5.4.4-16): Mean Bile Flow in g/hr/kg from F344 Du/Crl and Crl:CD(SD) Rats.

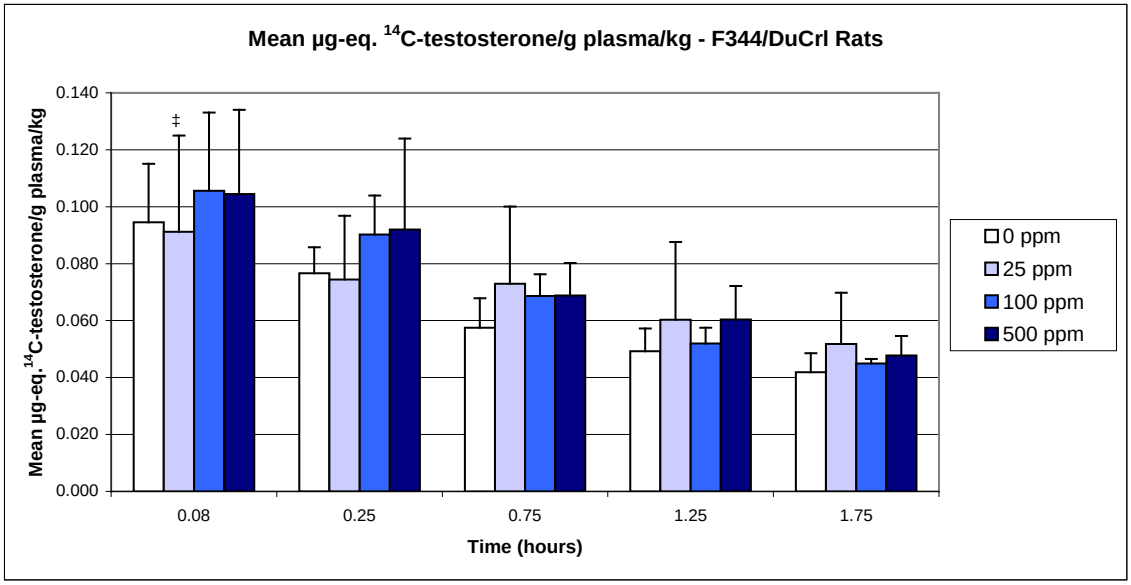


Figure 4.10.3.1.Study 10.17 (DAR Figure 6.5.4.4-17): Mean µg-eq. ^{14}C-testosterone/g plasma/kg from F344/DuCrl and Crl:CD(SD) Rats

Table 4.10.3.1.Study 10.22 (DAR Table 6.5.4.4-22). Summary of evidence for absence of increased testosterone biliary elimination by sulfoxaflor.

Test	Test System	Study design	Result	Report ref. (Study ID)
***In vivo* tests**				
Leydig cell tumour MoA study	F344 and Crl:CD(SD) rats	0, 25, 100 or 500 ppm for up to 8 wks	No effect of treatment on biliary elimination of testosterone No ↓T at any timepoint (2, 4, 8 wks)	Rasoulpour *et al.*, 2010a

Two-generation reproduction study	Rat/CD	0, 25, 100, 400 ppm 208 for ~ 10 weeks prior to breeding, through breeding, gestation and lactation for 2 generations M: 0, 1.5-1.7, 6.1-6.9, 24.6-28.1 (range of doses for F1 ad F2 males) F: 0, 1.6-2.1, 6.6-8.4, 26.8-34.0 mkd (range of doses for F1 and F2 females over course of treatment)	No indicators of oestrogenic or anti-oestrogenic effects: no treatment-related effects on female reproductive organ weights, reproductive histopathology, vaginal patency, oestrus cyclicity, mating, fertility, time to mating, or gestation length	Rasoulpour *et al*., 2010b (091023)

Based on the weight-of-evidence (table 6.5.4.4-22), considering both direct data which shows sulfoxaflor has no effect on biliary elimination of testosterone and there is no transient decrease in testosterone and indirect data generated from the two-generation reproduction study, an increase biliary excretion of testosterone elimination MoA is not a plausible alternative MoA for the Leydig cell effects seen in F344 rats after two years of treatment with sulfoxaflor.

Table 4.10.3.1.Study 10.23 (DAR Table 6.5.4.4-23): Summary of evidence for absence of GnRH agonism by sulfoxaflor.

Test	Test System	Study design	Result	Report ref. (Study ID)
In vivo tests				

Two-generation reproduction study	Rat/CD	0, 25, 100, 400 ppm 208 for ~ 10 weeks prior to breeding, through breeding, gestation and lactation for 2 generations M: 0, 1.5-1.7, 6.1-6.9, 24.6-28.1 (range of doses for F1 ad F2 males) F: 0, 1.6-2.1, 6.6-8.4, 26.8-34.0 mkd (range of doses for F1 and F2 females over course of treatment)	No treatment-related effects on pituitary gland, anogenital distance, no effects on testis or accessory sex gland (i.e., prostate, seminal vesicle, and epididymis) weight or histopathology	Rasoulpour *et al*., 2010b (091023)
28-day toxicity study	Rat/F344	M: 0, 300, 1000, 2000, 3000 ppm (0, 24.8, 79.4, 155, 205 mkd) F: 0, 300, 1000, 2000, 3000 ppm (0, 26.5, 88.3, 170, 192 mkd)	No effect on pituitary gland weight or histopathology	Yano, 2009a (061170)
90-day toxicity study	Rat/F344	M: 0, 100, 750, 1500 ppm (0, 6.36, 47.6, 94.9 mkd) F: 0, 100, 750, 1500 ppm (0, 6.96, 51.6, 101 mkd)	No effect on pituitary gland weight or histopathology	Yano, 2009b (071057)
Oncogenicity study	Rat/F344	M: 0, 25, 100, 500 ppm (0, 1.04, 4.24, 21.3 mkd) F: 0, 25, 100, 750 ppm (0, 1.28, 5.13, 39.0 mkd)	No effect on pituitary gland weight or histopathology	Stebbins *et al.* 2010 (071187)

8) GnRH (LHRH) agonism – Not Plausible

A prototypical GnRH agonist, such as buserelin, would cause both reduced accessory sex

gland weights (due to negative feedback HPG-axis compensation) as well as cause histopathological effects in the pituitary gland, as this is the primary site of functional GnRH receptor expression. As mentioned previously there were no effects on accessory sex gland weights in the sulfoxaflor two-generation reproductive toxicity study as well as no treatment-related effects on the pituitary gland in any rat toxicity study including the rat two-year oncogenicity study.

Based on the weight-of-evidence (table 6.5.4.4-23), considering indirect data showing no effect on the pituitary gland, a GnRH agonism MoA is not a plausible alternative MoA for the Leydig cell effects seen in F344 rats after two years of treatment with sulfoxaflor.

Conclusion on consideration of alternative MoAs

A summary evaluation for the considered alternative MoAs is presented in table 6.5.4.4-24. Following consideration of the presented alternative MoAs it is concluded that there is sufficient evidence to exclude the alternative MoAs for sulfoxaflor promotion of LCTs in Fischer 344 rats.

F. Sulfoxaflor rodent leydig cell tumour human relevance framework.

Question 1. Is the weight of evidence sufficient to establish the MoA in animals?

The answer is yes. The MoA for sulfoxaflor-induced Fischer rat Leydig cell tumours is compatible with that described for dopamine agonists/enhancer-induced tumours. The available data for sulfoxaflor presented in this MoA/HRF provide evidence supporting MoA #9 in the form of decreased circulating Prl levels, with increased LH, along with decreased testis LHR gene expression. This MoA could operate through sulfoxaflor-mediated enhancement of dopamine release, potentially through agonism of central nicotinic acetylcholine receptors, which play a key regulatory role in dopamine release from dopaminergic neurons in the brain. As mentioned previously, sulfoxaflor is a weak agonist to the foetal rat muscle nAChR and the insect nAChR is the target of the insecticidal mechanism for sulfoxaflor. Based on these data, it is plausible that the LCT promotion seen in the rat chronic/carcinogenicity study was through subtle, but chronic, enhancement of dopamine release, and subsequent inhibition of prolactin release from the pituitary gland, ultimately leading to a dopamine agonism/enhancement LCT MoA in a uniquely susceptible animal model, the Fischer 344 rat. In addition, other possible MoAs were examined and evaluated to be unlikely based on analysis of the relevant data for sulfoxaflor.

Question 2. Can human relevance of the MoA be reasonably excluded based on fundamental qualitative differences in key events between experimental animals and humans?

The answer is yes. As previously discussed, this MoA/HRF was designed to evaluate the MoA for the increased size of Fischer rat LCT observed in the sulfoxaflor 2-year rat oncogenicity study at 100 and 500ppm, and increased incidence of bilateral LCT at 500ppm. The effect in question is subtle in nature and the background incidence of Fischer rat LCT is 75-100% in 2-year studies compared to 1-5% in CD rats, even less in CD-1, and orders of magnitude lower in ranges of 0.01 – 0.00004% for humans. These interspecies differences in background incidence are well

Table 4.10.3.1.Study 10.24 (DAR Table 6.5.4.4-24). Summary Evaluation for Other Possible MoAs

Alternative MoA	Example	Strength of Association	Consistency of Association	Specificity of Association	Dose-Response Concordance	Temporal Relationship	Coherence & Plausibility
Relevant to Humans							
1) Mutagenicity	Cadmium	- Negative for genotoxicity[#]	-	-	- No tumors at lower doses	- Late onset tumor type	- Coherence - Plausibility
Low Relevance to Humans							
2) Androgen receptor antagonism	Vinclozolin Flutamide	- No AR transactivation	- No evidence from apical endpoints in sub-chronic rat and mouse oncogenicity *in vivo* studies*	-	-	-	- Coherence - Plausibility
3) Estrogen receptor agonism/ antagonism	Diethylstilbestrol	- No ER binding or transactivation		-	-	-	- Coherence - Plausibility
4) 5-alpha-reductase inhibition	Finesteride	- No effect on 5αR gene expression in testes		-	-	-	- Coherence - Plausibility
5) Aromatase inhibition	Formestane Letrozole	- No aromatase inhibition		-	-	-	- Coherence - Plausibility
6) Reduced testosterone biosynthesis	Calcium channel blockers Cimetidine	- No effect on steroidogenic genes		-	-	-	- Coherence - Plausibility
7) Increased testosterone biliary elimination	Triazoles	- No increased T biliary elimination	-	-	-	-	- Coherence - Plausibility
No Relevance to Humans							
8) GnRH (LHRH) agonism	Buserelin		- No evidence from apical endpoints in 4/4* *in vivo* studies	-			- Coherence - Plausibility
9) Dopamine agonism/enhancement	**Mesulergine Bromocriptine**	**+ Moderate**	**+ Moderate**	**+ Moderate**	**+ Moderate**	**+/- Weak**	**+Coherence + Plausibility**

+ Indicates attribute present, - indicates attribute absent, +/- indicates equivocal. M=Male, F=Female

* Refers to studies: 28-day toxicity study in rats; 90-day toxicity study in rats; chronic/oncogenicity study in rats; 2-generation reproduction toxicity study in rats (where slight delay in BPS was seen); developmental neurotoxicity study in rats

[#] Bacterial mutagenicity (Ames) test, HGPRT, hypoxanthine-guanine phosphoribosyltransferase; RLCAT, rat lymphocyte chromosome aberration test; MNT, micronucleus test

understood, and result from quantitative and qualitative differences of Leydig cell response to hormonal stimuli. Rat Leydig cells contain >10-fold more LH receptors than humans, which confers greater sensitivity to slight changes in LH levels. In addition to this quantitative difference, rat, but not human, Leydig cells have both PrlR and GnRH receptors (GnRHR) on their surface. Stimulation of rat Leydig cells through both PrlR and GnRHR are a rat-specific mechanism by which LCT formation can occur. For PrlR involvement in LCT, dopamine agonists (e.g., muselergine) reduce Prl release by the anterior pituitary gland. This results in decreased binding of Prl to PrlR on Leydig cells, leading to downregulation of the LH receptor and transient reductions in testosterone production, which feeds back to induce LH release from the pituitary leading to Leydig cell stimulation and hyperplasia over time.

Given these differences between rat and human Leydig cells, independent experts have determined that "*..the weight of evidence suggests that human Leydig cells are quantitatively less sensitive than rat Leydig cells in their proliferative response to LH, and hence in their sensitivity to chemically induced LCTs. It can be concluded that no observable effect levels for the induction of LCTs in rodent bioassays provide an adequate margin of safety for protection of human health and that the data support a nonlinear mode of action (i.e., threshold response).*" Finally the authors conclude that "*..the data suggest that nongenotoxic compounds that induce LCTs in rats most likely have low relevance to humans under most exposure conditions because humans are quantitatively less sensitive than rats*" (*Cook et al., 1999*). A concordance analysis of the key events for a dopamine agonism/ehancement MoA is presented below in table 6.5.4.4-25.

Table 4.10.3.1.Study 10.25 (DAR Table 6.5.4.4-25): Concordance of Key Events for a Dopamine Agonism/Enhancement LCT MoA in Rodents and Humans

Key Event	Evidence in Rodents	Evidence in Humans

Increased dopamine release via nAChR agonism	Yes	Yes
Decreased serum prolactin levels	Yes	Yes
Downregulation of LHR gene expression in Leydig cells	Yes	No; unlike rat Leydig cells, human Leydig cells do not possess a prolactin receptor and there is no evidence of human Leydig cell tumours from dopamine agonist treatments for hyperprolactinemia or Parkinson's disease.
Transient decrease in serum testosterone levels	Yes	No; none reported
Increased serum LH	Yes	No; none reported
Promotion of Leydig cell tumourigenesis	Yes	No; based on epidemiological data

Question 3. Can human relevance of the MoA be reasonably excluded based on quantitative differences in either kinetic or dynamic factors between experimental animals and humans?

As human relevance of the experimental animal MoA can be reasonably excluded on the basis of qualitative differences in key events (Question 2); a quantitative assessment of kinetic or dynamic factors is not necessary. However, as described in the background section of this document, there are significant differences in the background incidence of LCT across species and strains, with Fischer 344 rats being the most and humans being the least, sensitive. The biological basis for these differences in susceptibilities is described in detail within the background section of this report and includes both qualitative and quantitative differences in the underlying biology between rat and human Leydig cells.

Reliability of the Study:

Statement of confidence in the evaluation. This MoA and Human Relevance Framework evaluation for sulfoxaflor-induced Leydig cell tumours in Fischer rats follows the guideline established for this process (*Sonich-Mullin et al., 2001; Cohen et al., 2003; Meek et al., 2003; USEPA, 2005; Boobis et al., 2007*). The extensive toxicological database for sulfoxaflor, including several focused *in vitro* and *in vivo* MoA experiments are high quality studies, which provide the necessary data to evaluate the MoA for sulfoxaflor-induced rodent Leydig cell tumours. Analysis of these data revealed a proposed hormone-based dopamine enhancement mode-of-action (MoA) through the following key events: 1) increased neuronal dopamine release via nicotinic acetylcholine receptor (nAChR) agonism, leading to 2) decreased serum prolactin levels, leading to 3) downregulation of luteinizing hormone (LH) receptor gene expression in Leydig cells, leading to 4) transient decreases in serum testosterone, leading to 5) increased serum LH levels, leading to 6) promotion of Leydig cell tumourigenesis. The subtle nature of the supportive data for this MoA is not surprising given the latency and subtle nature of the effects in question. The two findings that anchor the analysis to the dopamine enhancement MoA are the decreased serum prolactin levels and concomitant decrease in LHR gene expression. These findings are unique to the key event progression of this particular MoA.

The conclusion from this evaluation is that the LCT promotion observed in the oncogenicity study was through a subtle, but chronic, dopamine enhancement MoA in a uniquely susceptible animal model, the Fischer 344 rat. The data for sulfoxaflor are judged with a moderate degree of confidence to adequately explain the promotion of Fischer rat Leydig cell tumours following chronic dietary administration of sulfoxaflor, and judged with a very high degree of confidence to support a hormonally-mediated, threshold based, nonlinear MoA.

Other possible MoAs for Leydig cell tumourigenesis as described (*Cook et al., 1999*) have been evaluated with respect to sulfoxaflor. This in-depth analysis of alternative MoAs revealed direct and/or indirect data to refute the eight other known possible MoAs to develop rodent LCTs. Importantly, very strong *in vitro* and *in vivo* data exist to refute a genotoxic mechanism. Taken together, all other MoAs have been dismissed for sulfoxaflor induced LCT because they lack plausibility and coherence with the significant data from the mechanistic and guideline toxicity studies on sulfoxaflor.

Identification of data gaps. Due to the subtle nature and long latency for the effects in question,

in combination with feedback compensation by the HPG axis, it is not surprising that the hormone and associated key events are transient during short-term studies. Therefore, these are not considered data gaps as it is more a function of the underlying biology. However, there are three data gaps identified during the analysis of this MoA, which are 1) lack of direct data for Key Event #1, 2) lack of direct data for Key Event #4, and 3) incomplete demonstration of key events at the 100ppm dose level.

Key Event #1 within this MoA is increased dopamine release via agonism on central dopaminergic neurons nAChRs. As outlined within the analysis of this key event, due to a combination of limited characterisation of nAChRs within the median eminence and technical and biological complexity of measuring neurotransmitters within the hypothalamic-hypophyseal portal vein system, there are no direct data supporting Key Event #1. However, as there is a direct inverse correlation between prolactin and dopamine, the decrease in serum prolactin levels within Key Event #2 can be used as indirect support for Key Event #1. Results from the *in vivo* dopamine microdialysis study indicate that sulfoxaflor may increase extracellular dopamine levels in the mediobasal hypothalamus, an area near to the median eminence and acting as a surrogate target to the actual presumed target of sulfoxaflor *in vivo* – the tuberoinfundibular (TIDA) system (section B.6.5.4.2; *Rowley & Heal, 2011*).

Key Event #4 within this MoA is a transient decrease in serum testosterone levels. Under the conditions of the LCT MoA study, there were no measurable decreases in serum testosterone; however, as described within the analysis of Key Event #4, the delay in balanopreputial separation from the two-generation reproductive toxicity study supports a transient decrease in testosterone. While these data are supportive and provide strong indirect evidence on a testosterone effect, there are no hormone measurement data that show a decrease in serum levels of testosterone.

Finally, while there are data supporting the MoA at 500ppm, no precursor key events were observed at 100ppm. A dose-response relationship for these apical end point effects existed with increased testis size and increased incidence of bilateral tumours at 500ppm. Due to the high background incidence of these tumours in Fischer rats, the lack of precursor key events for this subtle, hormone-based MoA at the lower 100ppm dose level is not surprising, especially given the transient and compensatory nature of hormone regulation in the HPG axis

Implications for risk assessment. Sulfoxaflor causes promotion of Leydig cell tumours (LCT) in a Fischer rat carcinogenicity study. The effect in question is subtle in nature and the background incidence of Fischer rat LCT is 75-100% in 2-year studies compared to 1-5% in CD rats, even less in CD-1 mice, and orders of magnitude lower in ranges of 0.01 – 0.00004% for humans. These interspecies differences in background incidence are well understood, and result from quantitative and qualitative differences of Leydig cell response to hormonal stimuli. Rat Leydig cells contain >10-fold more LH receptors than humans, which confers greater sensitivity to slight changes in LH levels. In addition to this quantitative difference, rat, but not human, Leydig cells have both PrlR and GnRH receptors (GnRHR) on their surface. Stimulation of rat Leydig cells through both PrlR and GnRHR are a rat-specific mechanism by which LCT formation can occur. For PrlR involvement in LCT, dopamine agonists (e.g., muselergine) reduce Prl release by the anterior pituitary gland. This results in decreased binding of Prl to PrlR on Leydig cells, leading to downregulation of the LH receptor and transient reductions in testosterone production, which

feeds back to induce LH release from the pituitary leading to Leydig cell stimulation and hyperplasia over time.

Given these differences between rat and human Leydig cells, independent experts have determined that "*that human Leydig cells are quantitatively less sensitive than rat Leydig cells in their proliferative response to LH, and hence in their sensitivity to chemically induced LCTs. It can be concluded that no observable effect levels for the induction of LCTs in rodent bioassays provide an adequate margin of safety for protection of human health and that the data support a nonlinear mode of action (i.e., threshold response).*" Finally the authors conclude that "*the data suggest that nongenotoxic compounds that induce LCTs in rats most likely have low relevance to humans under most exposure conditions because humans are quantitatively less sensitive than rats*".

Taken together, the promotion of Fischer rat LCT observed in the oncogenicity study has a MoA that is hormonally-mediated and threshold-based, and would be considered to have no relevance to humans due to qualitative and quantitative differences between rat and human Leydig cells. On this basis, the Fischer rat Leydig cell tumours associated with administration of high dose level of sulfoxaflor would not pose a cancer hazard to humans. Based on this hazard assessment for the sulfoxaflor-induced LCT effect, a margin of exposure risk assessment based on the chronic reference dose (cRfD) would be protective of human health.

Reliability of the study:

Even though the background incidence of Leydig cell tumours is incredibly high in the Fisher 344 strain of rat, the opinion is that there is sufficient data in the longterm / carcinogenicity study that sulfoxaflor has a treatment related effect on the Leydig cell tumours observed at the end of the combined chronic/carcinogenicity study in F344 rats. There are clear indications of greater tumour burden with increased testicular weights, extensive secondary effects due to tumour mass and increased bilateral incidences.

It is considered that the proposed mode of action (MOA) for the Leydig cell tumours is plausible considering all of the data submitted. A MOA based on weak but sustained secondary dopamine release based on agonism of central nicotinic acetylcholine receptors on the cell bodies of the tuberoinfundibular (TIDA) neurones in the arcuate nucleus of the hypothalamus is considered plausible. A slight increase in dopamine concentration in the hypothalamic-hypophysial portal vessels would impact on the lactotrophs in the anterior lobe of the pituitary gland by further inhibiting prolactin secretion. Downstream consequences of reduced plasma prolactin would appear to be species specific to the rat (and mouse?) due to distinct molecular differences between rat and human Leydig cells. Publicised literature has well documented cases of Leydig cell tumours in rats upon treatment with dopamine agonists, there is little to no information to suggest that humans on dopamine agonist treatment are susceptible to an increased incidence of testicular tumours though there are perturbations in plasma testosterone response to hCG challenge (*Oseko et al., 1991*). Additionally, *Oseko* and colleagues showed that there were no significant changes to plasma LH in human males treated with bromocryptine while there were significant reductions in plasma prolactin (*Oseko et al., 1993*).

There are however uncertainties and inconsistencies in the results from the various studies:

- The postulated MoA includes decreased testosterone as a key event. There was a delay in preputial separation noted in males in the two-generation reproduction study that indicates a possible decrease in testosterone. However, no measurable decreases in serum testosterone were seen in the Leydig cell tumour MoA study.

- Although there were changes present related to specific key events (decreased prolactin and LH receptor expression), the changes observed were subtle and presented a weak dose response. Additionally, the only statistically significant changes were seen in dopamine release and LH levels and the LH changes were only seen at the tumourigenic dose.

- The concentrations used to evaluate dopamine release were based on the plasma concentration of rats after 12 months; therefore, it is unclear whether the concentrations are reflective of plasma concentration in rats after 24 months.

- No Leydig cell hyperplasia or proliferation was observed after sulfoxaflor exposure.

- Dopamine agonist positive controls would have helped in the interpretation of the results in some of the MoA studies.

Overall, the weight of evidence for the Leydig cell MoA suggests Sulfoxaflor causes further promotion of Leydig cell tumours (LCT) in the Fischer male rat. In conjunction with external evidence it would appear that interspecies differences in the background incidence of LCTs are well understood, and result from quantitative and qualitative differences of Leydig cell response to hormonal stimuli. Consequently the considersation is that the MoA presented for sulfoxaflor has no relevance to humans and that sulfoxaflor is unlikely to pose a cancer hazard to humans.

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Study 11: Human Relevance Framework for Preputial Gland Carcinoma. DAR Section B.6.5.4.5.

Similarly, a Human Relevance Framework (HRF) analysis of preputial gland tumours was also submitted but there was no direct experimental investigation into this effect. Questions remain as to the actual incidence of this tumour type because only animals with palpatable masses were histologically evaluated in the long term carcinogenicity study so that the results cannot be interpreted as a proportion of the total number of animals in each treatment group. There is insufficient evidence regarding this effect but because humans do not have a preputial gland or equivalent, the opinion is that this finding may have no relevance to humans, per se. The conclusion from the framework analysis was that the observed sulfoxaflor-induced promotion of preputial gland tumours is considered likely to be secondary to the LCTs, and of little human relevance. It was postulated that the effect is a consequence of resetting the HPG axis to a slightly higer level of activity resulting in a chronic increase in testosterone production but there is no direct evidence for this mode of action.

The rat preputial gland is testosterone dependent for both its proliferation and differentiation (Miyake et al., 1994; Ponmanickam et al., 2010). While Miyake et al., (1994) make the point that androgen receptor mRNA is most abundant in the mid-differentiation sebocytes, rather than the less differentiated and more proliferative precursor cells, it is clear from several studies that testosterone provides a key proliferative signal to the rat preputial gland (Freinkel, 1963; Ponmanickam et al., 2010). Data to support an increase in serum testosterone due to resetting of the HPG axis mostly comes from the peer-reviewed literature with other dopamine agonists/enhancers.

In the Leydig cell tumour MoA study (section B.6.5.4.1; Rasoulpour, 2010a), serum testosterone measurements were done in both Fischer 344 and Crl: CD (SD) rats after 2, 4 and 8 weeks of sulfoxaflor exposure. Statistically significant increases (↑84%; *p<0.05) in testosterone were noted in SD rats at the high dose of 500ppm after 2 weeks. Statistically significant increases

were also seen at 25ppm (↑123%; *p<0.05), but not at the mid dose of 100ppm (↑83%). In F344 rats, increases in testosterone were seen at 25ppm and 500ppm after 2 weeks. However, decreases were seen at 100ppm. Increases in testosterone were also seen in both strains of rat after 4 and 8 weeks at ≥ 25ppm, the changes were not statistically significant.

Report: K. E. Stebbins, R. J. Rasoulpour and K. Boekelheide. (2011). XDE-208 (sulfoxaflor): mode of action and human relevance framework analysis of preputial gland carcinomas in the two-year f344/ducrl rat carcinogenicity assay. Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan, 48674. Unpublished.

Report No.: Study ID: 110175.

Dates: 2011

Guidelines: Non-guideline. Not required for EU dossier submission. It is however a useful summary of the data regarding sulfoxaflor exposure and preputial gland tumour incidence and relevance to man. This is submitted as a supplementary study/assessment in support of this DAR.

GLP: Not applicable.

Deviations: None. This is an acceptable overview of all the data presented thus far in section B6.5 as pertains to sulfoxaflor-induced preputial gland tumours in rodents and the toxicological relevancy of this effect to man. There is quite a bit of overlap with the information contained within section B.6.5.4.4. As in many other sections of this DAR, a reference list is compiled at the end of each subsection relating to the peer reviewed literature for the endocrine effects thought to be responsible for the mode of action.

Deficiencies: None. General discussion document.

Abstract: Sulfoxaflor caused a marginal increased incidence of preputial gland carcinoma, which did not reach statistical significance, in the F344/DuCrl rat carcinogenicity study. This effect was limited to the high dose level of 500ppm, with a no-observed-effect level of 100ppm (4.24 mg/kg bw/ day). The proposed mode-of-action (MoA) for this effect includes the following Key Events (KE), and is not relevant to humans:

- Agonism, via nicotinic acetylcholine receptors, to dopaminergic neurons in the hypothalamus resulting in increased dopamine release.
- Dopamine-mediated inhibition of prolactin release from the anterior pituitary resulting in reduced serum prolactin levels.
- Reduced stimulation of prolactin receptors on Leydig cells resulting in reduced luteinizing hormone (LH) receptor density on Leydig cells (human Leydig cells do not have functional prolactin receptors and hence the sequence of events beyond this step cannot occur in humans).

- Reduced LH receptor density leads to transiently reduced testosterone production by Leydig cells.
- Reduced serum testosterone levels stimulates increased production of LH from the pituitary
- The continuous drive of increased dopamine release leads to a 'resetting' of the hypothalamic-pituitary-gonadal (HPG) axis to a slightly higher level of activity and hence higher testosterone production.
- The slightly higher testosterone level stimulates preputial gland proliferation which, over a lifetime, promotes normal spontaneous tumourigenesis in the rat preputial gland.

Table 4.10.3.1.Study 11.1 (DAR Table 6.5.4.5-1: Sulfoxaflor): Temporality and dose response for MoA key events related to male F344/DuCrl rat preputial gland carcinoma.

Temporal →

Dose ↓

Dose (ppm)	Key Event 1	Key Event 2	Key Event 3	Key Event 4	Key Event 5	Key Event 6	Key Event 7
	Increased dopamine release via nAChR agonism	Decreased serum prolactin levels	Downreg of LHR gene expression in Leydig cells	Transient decreased serum testosterone levels	Increased serum LH levels	Reset of HPG axis / increased serum testosterone	Promotion of preputial gland tumors
25		-	-	-	-		-
100		-	-	-	-		-
400				+*			
500	+	+	+	-	+	**	+
	+ indicates effect present, - indicates effect absent, blank cell indicates no data. * indicates indirect data from delay in balanopreputial separation data. **indicates no direct data, but supportive evidence in the literature.						

Overall, the weight of evidence (WoE) supports no relevance of preputial gland carcinomas for human health risk assessment because:

- The MoA for sulfoxaflor-induced preputial gland carcinoma is not relevant to humans.
- Sulfoxaflor has no indication of genotoxicity from *in vitro* and *in vivo* assays for mutagenicity or clastogenicity.
- Humans do not have an anatomic equivalent to rodent preputial glands.
- There were no effects in the female rat correlate to the preputial gland (clitoral gland).
- Even at higher doses, there were no effects in CD-1 mouse preputial glands, clitoral glands, or other sebaceous glands (skin, Zymbal's gland).
- There were no effects in other sebaceous glands (skin, Zymbal's gland) in male or female F344/DuCrl rats.

In summary, the MoA for the sulfoxaflor's promotion of preputial gland carcinoma is dopamine enhancement, which is the MoA responsible for the Leydig cell tumour promotion and its associated effects on the epididymides and accessory sex glands of F344/DuCrl rats. This is a hormonally-mediated, threshold based, nonlinear MoA. As indicated by published literature (*Cook et al., 1999*), this MoA is not relevant to humans.

(A) Introduction:

In a two-year rat carcinogenicity study with sulfoxaflor, male F344/DuCrl rats given 500ppm sulfoxaflor had a marginal, not statistically significant, increased incidence in preputial gland carcinoma (PGC) (section B.6.5.1.1; *Stebbins et al., 2010*). The incidence of PGCs within this study was 5/50, 7/50, 7/50, and 10/50 in males given 0 (controls), 25, 100, or 500ppm, respectively. The marginal increase in high-dose males was conservatively interpreted to be treatment related because the incidence was slightly higher than the historical control range of 0 to 6 PGCs in the four most recent previously conducted F344/DuCrl dietary carcinogenicity studies at the same laboratory. The study where six PGCs occurred was conducted contemporaneously with the sulfoxaflor carcinogenicity study. For sulfoxaflor, the no-observed-effect level for PGCs was the intermediate dose of 100ppm, since the incidence of seven PGCs at 25 and 100ppm was interpreted to be comparable to the historical control range of PGCs.

The analysis of the relevant toxicity and MoA studies of sulfoxaflor herein provides the context to evaluate the proposed MoA for PGC, which is a shared MoA for Leydig cell tumours (LCT) promotion also observed in the sulfoxaflor two-year F344/DuCrl rat carcinogenicity study. The key features of the Guidelines for Carcinogen Risk Assessment (USEPA, 2005) were applied to the assessment of the preputial gland carcinomas in this study, as well as the MoA-Human Relevance Framework (*Cohen et al., 2003; Meek et al., 2003; USEPA, 2005; Boobis et al., 2007*). In addition, the relevant literature on the underlying biology and molecular mechanisms was reviewed, including the National Toxicology Program (NTP) database. Included in this assessment is a discussion of the promotion of Leydig cell tumours (LCTs) that occurred in male F344/DuCrl rats, since a consequence of mode of action (MoA) for LCTs is the enhanced development of preputial gland carcinomas (PGCs) through a non-genotoxic mechanism. This analysis indicates that the marginal PGC effect seen in the rat chronic/carcinogenicity study was

through subtle, but chronic, enhancement of dopamine release, subsequent inhibition of prolactin release from the pituitary gland that alters hormonal balance and resets the hypothalamic-pituitary-gonadal (HPG) axis. This results in slight continuous elevation in serum testosterone, which is the likely mechanism for preputial gland hyperplasia, and ultimately leads to a dopamine agonism/enhancement MoA in a tissue (preputial glands) not found in humans. This MoA is considered to have no relevance to humans due to qualitative and quantitative differences between human and the F344/DuCrl rat Leydig cells. In addition to providing data to support or refute specific LCT MoA, the observation of hormone level alterations in the sulfoxaflor LCT MoA study (*Rasoulpour et al., 2011*) clearly support a hormonally-mediated, and thereby threshold, nonlinear mode-of-action.

(B) Biology of the preputial gland:

The rat preputial gland is a modified sebaceous gland that has a role in pheromone secretion and sexual behavior (*Ponmanickam et al., 2010*). There is no anatomical equivalent of the rat preputial gland in the human (*Monro and Mordenti, 1995*). The rat preputial gland is testosterone dependent for both its proliferation and differentiation (*Miyake et al., 1994; Ponmanickam et al., 2010*). While *Miyake et al.*, (1994) make the point that androgen receptor messenger ribonucleic acid (mRNA) is most abundant in the mid-differentiation sebocytes, rather than the less differentiated and more proliferative precursor cells, it is clear from several studies that testosterone provides a key proliferative signal to the rat preputial gland (*Freinkel, 1963; Ponmanickam et al., 2010*).

There are unique features that distinguish the preputial gland from the other secondary sex organs in the rat, notably the presence of type 1 5α-reductase instead of the type 2 5α-reductase found in prostate (*Deplewski et al., 1997*). These different types of 5α-reductase have different substrate specificities and activities (*Deplewski et al., 1997*). As modified sebaceous glands, the rat preputial gland also shares many of the receptor and signaling attributes of this cell type (*Zouboulis, 2009*), characteristic of its epidermal origin as distinct from the endodermal origin of the prostate. This difference in developmental origin likely explains the differential responses to various hormones shown by the preputial gland compared to other secondary sex organs such as the prostate. An example of the sensitivity of the preputial gland to hormonal stimulation is its response to α-melanocyte stimulating hormone, a member of the adrenocorticotropic hormone family, and other pituitary hormones (*Thody et al., 1976; Thiboutot et al., 2000*).

Preputial gland tumours are mostly of acinar origin, although they may arise from the ducts, and are commonly found in control F344/DuCrl rats (*Mitsumori and Elwell, 1988*). They tend to occur toward the end of life. They most likely arise through a characteristic hyperplasia to adenoma to carcinoma sequence, since hyperplasia appears to be a preneoplastic rather than regenerative response. The diagnosis of carcinoma instead of adenoma is largely based on size and cytological features (*Maronpot et al., 1988*).

The spontaneous incidence of preputial gland tumours varies among different strains of rats, with a relatively high incidence in F344/DuCrl rats. An analysis of the National Toxicology Program (NTP) database revealed a statistically significant correlation between sexes for the occurrence of preputial gland and clitoral gland neoplasms following chemical exposure, and no correlation across species (comparing rat to mouse) for this tumour type (*Haseman and Lockhart, 1993*).

The occurrence of preputial gland tumours is significantly affected by diet, as demonstrated by the reduced incidence of this tumour type following the change in 1994 to the NTP-2000 diet in the NTP 2-year carcinogencity assays (*Haseman et al., 2003*).

Sulfoxaflor induces a marginal increase in preputial gland carcinoma.

In the F344/DuCrl rat 2 year carcinogenicity study of sulfoxaflor (section B.6.5.1.1; *Stebbins et al., 2010*), there was a marginal increase in the incidence of PGCs in high-dose (500ppm) males compared to controls. A point of concern in interpreting the incidence of PGCs in this study is determining the appropriate number of animals in which the preputial gland was adequately examined. The regulatory guidelines that were followed in this study (OECD guideline 453, 1981; USEPA OPPTS 870.4300, 1998; EEC Part B, Combined chronic toxicity/carcinogenicity test, Directive 87/302/EEC., 1988; and JMAFF Combined Chronic Toxicity/Oncogenicity Study, 2000) do not require the preputial gland to be preserved for routine histopathological examination. Therefore, in accordance with the guidelines, only the preputial glands with grossly observed lesions from controls and treatment groups were examined microscopically. The most conservative approach to determine the percent incidence in each dose group would be to use the number of preputial glands examined microscopically as the denominator. This would result in a 63% incidence of PGCs in the control group (5 carcinomas in 8 preputial glands examined), and a 100% incidence in the high-dose group (10 carcinomas in 10 preputial glands examined). This approach results in a highly inaccurate estimate of the true incidence of PGCs. It should be noted that in the NTP historical control database from 2-year F344/DuCrl rat carcinogenicity studies conducted in the past 23 years, the mean incidence of PGCs was approximately 1.4 to 3.0% (table 6.5.4.5-2). This shows how implausible it would be to accept incidences of 63 to 100% PGCs in the sulfoxaflor 2-year rat study.

The definitive number of proliferative preputial gland lesions (hyperplasia, adenoma or carcinoma) in the sulfoxaflor study will never be known since only preputial glands with grossly evident lesions were preserved at necropsy. However, it is our assertion that the number of PGCs (5, 7, 7, and 10 at doses of 0, 25, 100, and 500ppm, respectively) are highly accurate values for all of the rats on this study. The following points support this assertion:

- All rats were given a hand-held physical examination once each week throughout the 2-year study. The examination included manual palpation of the preputial gland area. All preputial gland swellings that were detected during the in-life physical examinations were recorded for date of occurrence, size and physical features. Preputial gland swellings of significant size and duration were intermittently evaluated by board certified veterinary pathologists during the in-life phase of the study. This high level of supervision concerning preputial gland in-life swellings makes it highly unlikely that any carcinomas of the preputial glands were undetected.
- All rats (moribund, spontaneous deaths, and terminal sacrifice) were necropsied by a board certified veterinary pathologist. The necropsies of all animals always included macroscopic examination of the preputial gland area. As with the in-life inspections, this level of expertise by experienced veterinary pathologists gives

assurance that few, if any, proliferative lesions of the preputial gland would have gone undetected.

- The sulfoxaflor 2-year carcinogenicity study had 5/50 control group rats with PGCs, and a contemporaneous 2-year carcinogenicity study had 6/50 control group rats with PGCs. These numbers are slightly higher than the recent mean NTP historical control values of 1.4 to 3% (table 6.5.4.5-2). This indicates that the in-life and necropsy procedures of the laboratory that conducted the sulfoxaflor study were highly effective in their ability to detect proliferative lesions of the preputial gland.
- PGCs are rapidly growing neoplasms (*Maronpot et al., 1988*), and it is reasonable to expect that all of these tumours would have been detected grossly.
- There was no treatment-related effect on mortality at any dose level in the sulfoxaflor two-carcinogenicity study. Therefore, all animals that died spontaneously or were sacrificed moribund prior to the end of the study should be included in determining the number of animals adequately assessed for the presence of preputial gland proliferative lesions.

Therefore, based on the points above, the most plausible, and accurate, number to use in determining the percent incidence of PGCs is all 50 animals per dose group from the 24-month carcinogenicity phase of the study. The percent incidences of PGCs would be 10% (5/50), 14% (7/50), 14% (7/50), and 20% (10/50) in male rats given dietary concentrations of 0, 25, 100 or 500ppm sulfoxaflor, respectively.

Preputial gland carcinoma incidence

An analysis of the NTP database for the ability to detect preputial gland lesions when triggered by the presence of a gross lesion alone as compared to required examination of all glands (i.e. n=50/group) shows that carcinomas are found with almost equal incidence using these differing necropsy approaches, while adenomas are more often found with mandatory assessment (table 6.5.4.5-2). These data indicate that essentially all of the rapidly growing preputial gland carcinomas were likely to have been discovered by gross examination at necropsy in the 2 year carcinogenicity assay of sulfoxaflor. This issue of the 'true' incidence of the preputial gland tumours is presented later.

Table 4.10.3.1.Study 11.2 (DAR Table 6.5.4.5-2): Incidence of preputial gland tumors detected in control F344/DuCrl rats from NTP carcinogenicity studies

	Adenoma	**Carcinoma**	**Other**
Grossly abnormal examined only (1988-1992)[a]	3.46%	2.90%	0.21%
All examined (1989-1992)[b]	8.80%	2.99%	0.08%
All examined (2006-2011)[c]	3.69%	1.37%	0.08%

[a]n=25; [b]n=25; [c]n=25; Incidence was determined by adding up the number of lesions in all

studies and dividing by the total number of animals.

(C) Sulfoxaflor: rodent preputial gland carcinoma proposed mode of action:

Since sulfoxaflor is not genotoxic, a non-genotoxic MoA for induction of preputial gland tumours must be invoked. On this basis, and consistent with the sulfoxaflor Leydig cell tumour (LCT) MoA, the logical and parsimonious explanation for preputial gland tumour induction is a shared MoA responsible for LCT induction associated with alterations in signaling by the hypothalamic-pituitary-gonadal axis.

The proposed MoA (figure 6.5.4.5-1) for promotion of PGCs by sulfoxaflor includes the following Key Events, and is not relevant to humans:

1. Agonism, via nicotinic acetylcholine receptors, to dopaminergic neurons in the hypothalamus resulting in increased dopamine release.
2. Dopamine-mediated inhibition of prolactin release from the anterior pituitary resulting in reduced serum prolactin levels.
3. Reduced stimulation of prolactin receptors on Leydig cells resulting in reduced LH receptor density on Leydig cells (human Leydig cells do not have functional prolactin receptors and hence the sequence of events beyond this step cannot occur in humans).
4. Reduced LH receptor density leads to transiently reduced testosterone production by Leydig cells.
5. Reduced serum testosterone levels stimulates increased production of LH from the pituitary.
6. The continuous drive of KE #1 above, leads to a ‘resetting’ of the HPG axis to a slightly higher level of activity and hence higher testosterone production.
7. The slightly higher testosterone level stimulates preputial gland proliferation which, over a lifetime, promotes normal spontaneous tumourigenesis in the rat preputial gland.

The relevant experimental data for evaluation of the sulfoxaflor-induced rodent PGC MoA and human relevance includes guideline short-term/sub-chronic studies in the rat (28-day and 90-day), the two-generation reproductive toxicity study in rats, oncogenicity studies in the rat and mouse, as well as specific *in vivo* and *in vitro* Leydig cell MoA studies. These data will be presented in more detail during the evaluation of the MoA. During the MoA analysis, it is important to note that the apical end point finding is a marginal increased incidence in PGC at 500ppm, which did not reach statistical significance but was conservatively considered treatment-related. Given the magnitude of this effect, the stimulation of preputial gland proliferation and carcinoma likely resulted from a combination of the altered hormonal milieu in a senescent F344/DuCrl rat and promotion of this normal biological process by sulfoxaflor exposure. Therefore, for hormone-based MoAs, one would expect only subtle changes in young animals during shorter durations of exposure.

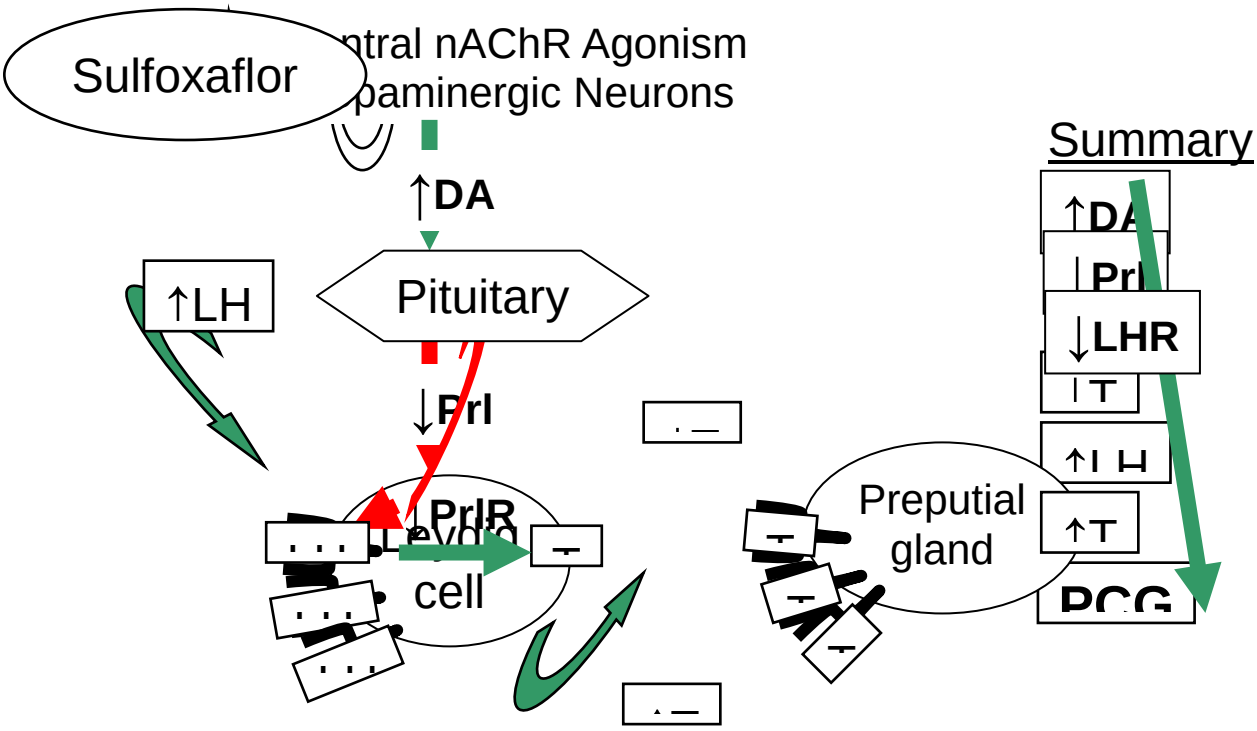


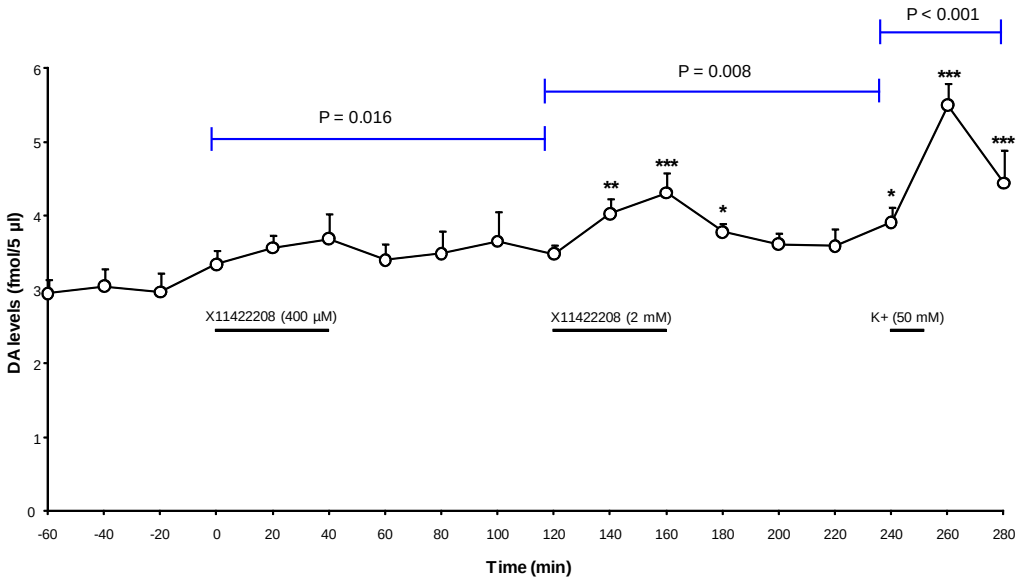
Figure 4.10.3.1.Study 11.1 (DAR Figure 6.5.4.5-1): Schematic of Proposed Dopamine-Enhancement MoA for Promotion of PGC

The proposed key events for the sulfoxaflor-induced rodent preputial gland carcinoma and the data that support these key events are described in subsequent sections in this document.

Key Event #1: Increased Dopamine Release via nAChR Agonism

The release of dopamine via central nAChR agonism by sulfoxaflor has recently been tested directly through a microdialysis experiment in freely moving rats (*Rowley and Heal, 2011*). The effects of reverse dialysis of sulfoxaflor on the extracellular concentration of dopamine in the mediobasal hypothalamus of rats was measured after infusion of 400µM (to replicate a concentration of 40 µM in the extracellular fluid of the mediobasal hypothalamus) and 2mM (to replicate a concentration of ~200µM in the extracellular fluid of the mediobasal hypothalamus) sulfoxaflor.

(a) Levels (fmol/5 µl)



(b) Percent of baseline

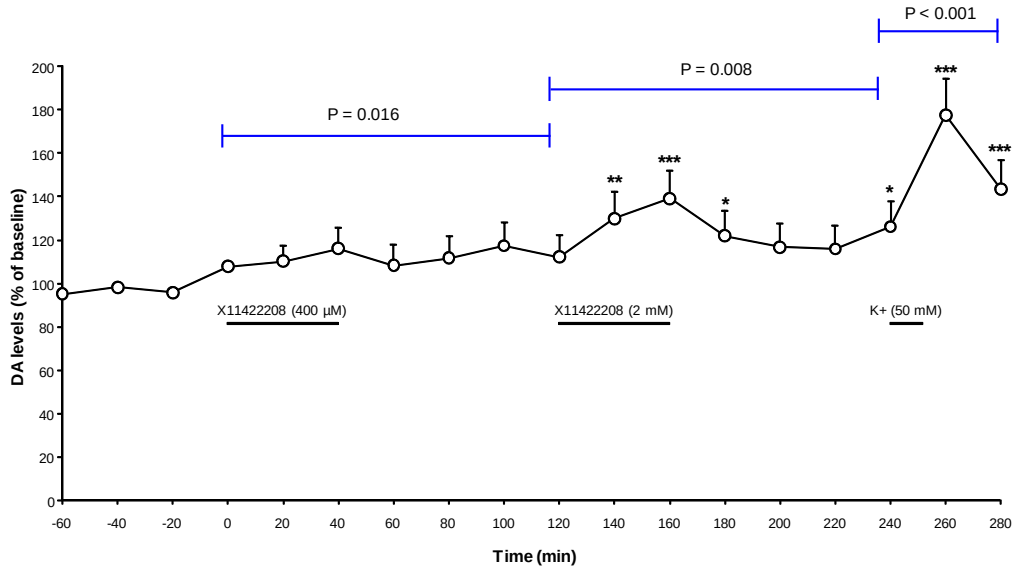


Figure 4.10.3.1.Study 11.2 (DAR Figure 6.5.4.5-2): Effects of sulfoxaflor infusion (400µM and 2mM) on extracellular levels of dopamine (DA) in the hypothalamus of the freely moving rat (levels (a) and % of baseline (b)). Results are adjusted means; n=7. SEMs are calculated from the residuals of the statistical model. Drug infusion is indicated by the horizontal bar. Data analysed by mixed linear model with animal as subject and treatment and treatment by time as factors followed by Williams' test (X11422208) and the multiple t-test (K^+). Significant differences versus baseline values are denoted by *p<0.05, **p<0.01 and ***p<0.001.

A depolarising pulse of K^+ ions produced a transient and sharply delineated increase in dopamine efflux indicating that these hypothalamic dopaminergic neurones were viable and normally responsive. Sulfoxaflor (400µM and 2mM) produced concentration related increases in the extracellular level of dopamine in the mediobasal hypothalamus (figures 6.5.4.5-2a and b). Relative to the magnitude of the effect of a depolarising pulse of K^+ ions, the increases evoked by sulfoxaflor were 25% at 400µM and 42% at 2mM.

The data support the hypothesis that through its nAChR partial agonist properties sulfoxaflor increases dopamine (DA) efflux from tuberoinfundibular dopamine (TIDA) neurons in the median eminence, and in turn, this effect is predicted to result in a decrease of prolactin secretion from the pituitary gland in the rat.

In addition, inhibition of prolactin release (Key Event #2) is primarily driven by dopamine release, the connection has been established with pharmaceutical dopamine agonists (*Prentice et al., 1992*), and this endpoint can be used as indirect evidence that sulfoxaflor causes this key event.

The insecticidal MoA for sulfoxaflor is binding to, and agonism on, the insect nAChR, and it has been shown that it is also an agonist to the rat foetal muscle subtype of the nAChR (*Rasoulpour et al., 2011*). It is plausible that the PGC promotion seen in the rat chronic/carcinogenicity study was through subtle, but prolonged, agonism at the central nAChRs within the median eminence causing release of dopamine and inhibition of prolactin release from the pituitary gland.

Key Event #2: Decreased Serum Prolactin Levels

In direct response to dopamine release from the hypothalamus to the anterior pituitary gland (Key Event #1), prolactin secretion to the systemic circulation is inhibited. Levels of serum prolactin were measured in the LCT MoA study (*Rasoulpour et al., 2011*) at 2, 4, and 8 weeks of exposure to 0, 25, 100, or 500ppm sulfoxaflor in F344/DuCrl rats. There was no effect of sulfoxaflor treatment on serum prolactin levels after two weeks of treatment; however, there was a 1.7-fold decrease in serum prolactin at 4 weeks in the 500ppm group with a concomitant 2-fold increase in serum LH levels (see Key Event #5), as shown in table 6.5.4.5-3 and figure 6.5.4.5-3. The effect on prolactin levels was not observed at the 8-week time point, which suggests compensation by the HPG axis. Note that the increase in baseline prolactin levels from 2-8 weeks in controls seen in this study is typical for young male rats (*Prentice et al., 1992*). Terminal blood samples were also collected from the LCT MoA study; however, because prolactin is a stress related hormone, levels across all groups were induced in response to carbon dioxide euthanasia associated stress: being 3-5-fold higher than in-life bleeds – the data can be seen in table 6.5.4.1-4 from section B.6.5.4.1. Table 6.5.4.5-4 shows the temporal and dose response for decreased prolactin levels from the LCT MoA study.

Due to the persistent compensatory nature of the hypothalamic-pituitary-gonadal (HPG) axis, coupled with the fact that chronic sulfoxaflor exposure for two years was required for only a marginal effect on PGC incidence in F344/DuCrl rats, it is not surprising that the changes observed in the hormone data are temporal in nature. Given the magnitude of this effect, the stimulation of preputial gland proliferation and carcinoma likely resulted from a combination of the altered hormonal milieu in a senescent F344/DuCrl rat and promotion of this normal

biological process by sulfoxaflor exposure. Therefore, for hormone-based MoAs, one would expect only subtle changes in young animals during shorter durations of exposure. These changes support a hormonally-mediated, and thereby threshold, nonlinear mode-of-action.

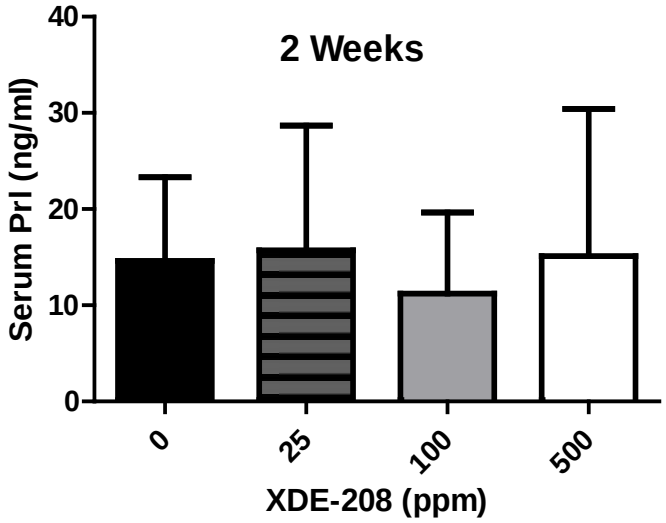
Table 4.10.3.1.Study 11.3 (DAR Table 6.5.4.5-3.) Sulfoxaflor: F344/DuCrl rat serum prolactin levels.	
Dose ppm	**Serum Prolactin (ng/ml)**
2-Week Treatment	
0	9.48
25	12.78
100	8.16
500	8.42
4-Week Treatment	
0	17.89
25	15.48
100	16.18
500	**10.39**
8-Week Treatment	
0	19.34
25	17.94
100	19.45
500	17.53
Bold type indicates treatment-related effect	

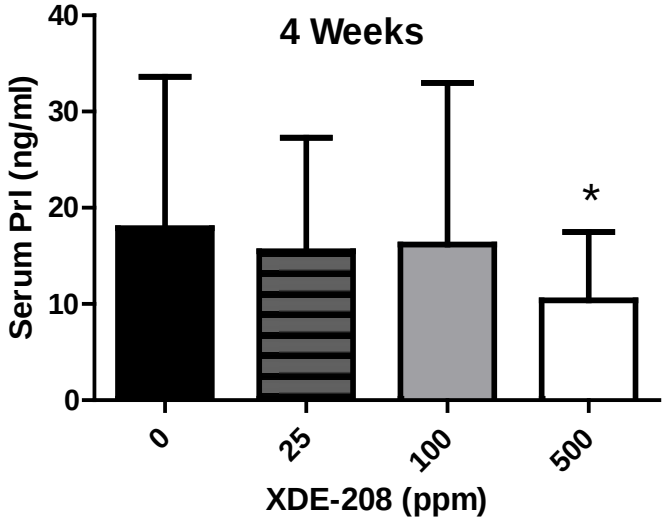
Table 4.10.3.1.Study 11.4 (DAR Table 6.5.4.5-4.) Sulfoxaflor Key Event #2: Temporal and Dose Response for Decreased Serum Prolactin Levels.

Temporal →

Dose

Dose ppm	2 weeks	4 weeks	8 weeks
0	100	100	100
25	135	86.5	92.8
100	86.1	90.4	100
500	88.8	**58.1**	90.6
Data are percentage of control values. **Bold** indicates treatment-related.			





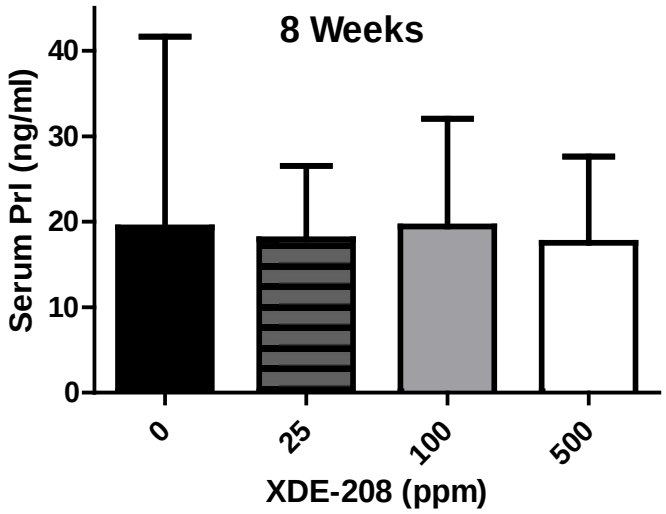


Figure 4.10.3.1.Study 11.3 (DAR Figure 5.5.4.5-3): Sulfoxaflor: F344/DuCrl Rat Serum Prolactin Levels (Mean ± S.D.)

Key Event #3: Downregulation of LH Receptor Gene Expression in Leydig Cells

In Key Event #3 of the dopamine agonism/enhancement MoA, lower serum prolactin levels (Key Event #2) in rats, but not humans, would lead to downregulation of LH receptor (LHR) gene expression (*Williams et al., 2007; Prentice et al., 1992*). Lower LHR expression would lead to a transient dip in testosterone production, leading to HPG-axis feedback stimulation and ultimately to increased LH release. Therefore if a dopamine enhancement MoA were operant, LHR gene expression would be decreased consistent with decreased circulating Prl hormone and increased LH. Real-time PCR was performed on 4- and 8-week isolated F344/DuCrl rat testis mRNA for the LH receptor (LHR) and prolactin receptor (PrlR) genes in order to determine if there was molecular concordance to the hormone data, which supported the dopamine enhancement MoA (*Rasoulpour et al., 2010a*).

Consistent with a dopamine enhancement MoA and the decreased Prl levels in the 4-week F344/DuCrl rat hormone data in Key Event #2, there was a ~1.6-fold dose-dependent decrease in LHR gene expression at the 4-week, but not 8-week, time point (table 6.5.4.5-4 and figure 6.5.4.5-4). In addition, there was a decrease in PrlR gene expression at the 4-week, but not 8-week, time point. While not statistically significant the magnitude of gene expression changes is consistent with the dynamic range of these genes in vivo and likely represents a biologically meaningful effect based on alterations in hormone levels. This conclusion is supported by a recent publication where administration of exogenous Prl to rats for 4-weeks resulted in a ~2-fold increase in LHR gene expression (*Williams et al., 2007*).

Table 4.10.3.1.Study 11.5 (DAR Table 6.5.4.5-5): Sulfoxaflor: F344/DuCrl Rat Testis LHR gene expression.

Dose ppm	LHR
4-Week Treatment	
0	1
25	-1.1
100	-1.1
500	**-1.6**
8-Week Treatment	
0	1
25	-1.3

100	1.2
500	1.1
Bold type indicates a treatment-related effect. Data presented as + or - fold-change of control	

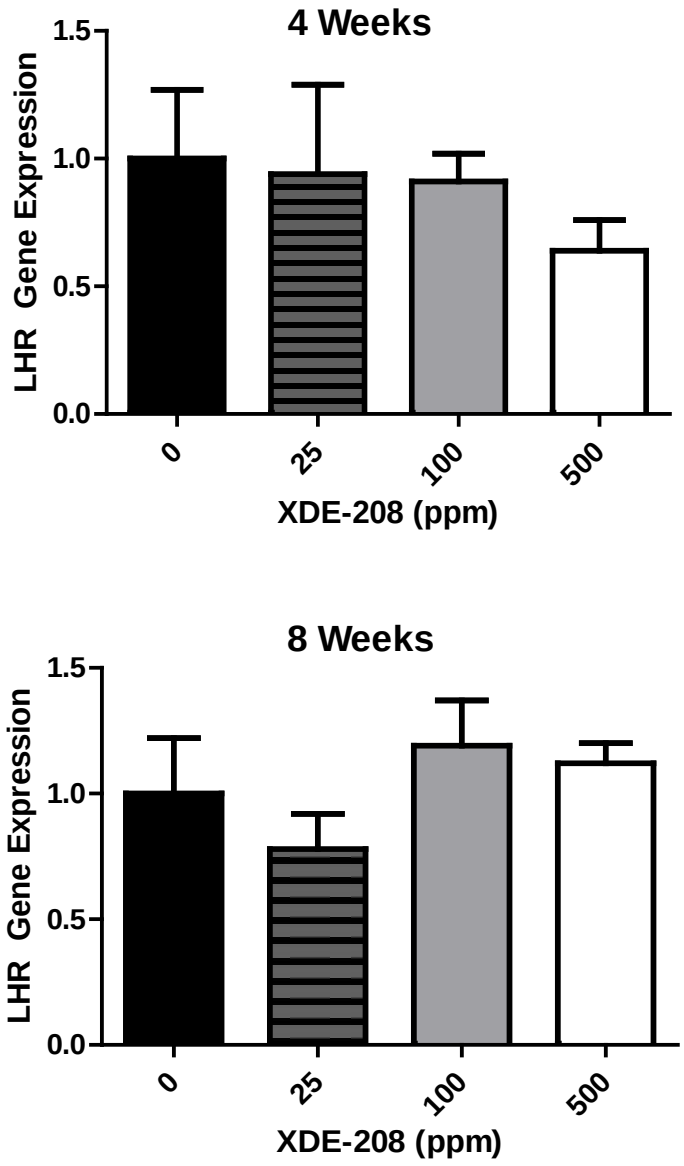


Figure 4.10.3.1.Study 11.4 (DAR Figure 6.5.4.5-4): Sulfoxaflor: F344/DuCrl Rat Testis LHR Gene Expression (Mean ± S.D.).

Consistent with the decrease in serum prolactin observed after 4-weeks of treatment with 500ppm sulfoxaflor, there was a biologically significant decrease in LHR gene expression at this dose level and time point. Also consistent with the prolactin hormone data – there were no differences from control of any other treatment group for LHR gene expression. Table 6.5.4.5-6 shows the temporal and dose response for downregulation of the LHR from the LCT MoA study.

Table 4.10.3.1.Study 11.6 (DAR Table 6.5.4.5-6): Sulfoxaflor Key Event #3: Temporal and dose response for decreased F344/DuCrl rat testis LHR gene expression.

Temporal →

Dose ↓

Dose ppm	4 weeks	8 weeks
0	100	100
25	94	78
100	91	119
500	**64**	112
Bold type indicates a treatment-related effect. Data are percentage of control values		

Key Event #4: Transient Decrease in Serum Testosterone Levels

Downregulation of the LHR in Key Event #3 leads to a transient decrease in serum testosterone levels in Key Event #4 (*Cook et al., 1999*). In LCT MoA experiments with the dopaminergic pharmaceutical agent mesulergine, serum testosterone levels were similar to controls at 2 weeks of treatment, slightly lower than controls at 4 weeks, returned to baseline by 10 weeks, and were elevated at 13 weeks (Key Event #6) (*Prentice et al., 1992*). Within the sulfoxaflor LCT MoA study, there were no measured decreases in serum testosterone levels at the 2-, 4-, or 8-week time point (*Rasoulpour et al., 2010a*), table 6.5.4.5-8. However, in the two-generation reproductive toxicity study, there was a treatment-related delay in balanopreputial separation (BPS) for male offspring in the high-dose group of 400ppm sulfoxaflor (*Rasoulpour et al., 2010b*), summarised in table 6.5.4.5-7 below. The process of BPS as a pubertal onset marker in a male rat is dependent on androgen levels, as testosterone injection to castrated rats is sufficient to induce BPS (*Korenbrot et al., 1977*). Therefore, in order for sulfoxaflor to induce a delay in BPS within the two-generation reproductive toxicity study, there had to be a decrease in testosterone levels (for at least some duration) during postnatal development. Further support for this statement is the fact that dopamine agonists such as bromocriptine induce a delay in male rat BPS (*Marty et al., 2001*).

This decrease in testosterone levels leading to a delay in BPS must have been a transient event as there were no effects on accessory sex gland weight, histopathology, or any other anti-androgenic finding in the adult males within the two-generation reproductive toxicity study that had a delay in BPS (*Rasoulpour et al., 2010a*). While many anti-androgenic molecules can cause a delay in BPS, these direct acting anti-androgens also cause a shortening in anogenital distance (AGD) at birth (*Wolf et al., 2000*).

Table 4.10.3.1.Study 11.7 (DAR Table 6.5.4.5-7.) Sulfoxaflor: Crl:CD(SD) rat balano-preputial separation	
Dose ppm	**Pubertal Parameter**
Age at Attainment (days)	
0	44.6
25	46.4
100	44.5
400	**47.0***
Body Weight at Attainment (g)	
0	253.6
25	265.8
100	250.3
400	**272.8**
* Statistically different from control mean by Dunnett's test, alpha = 0.05; **Bold type** indicates treatment-related effect	

Table 4.10.3.1.Study 11.8 (DAR Table 6.5.4.5-8.) Sulfoxaflor: F344/DuCrl rat serum testosterone levels	
Dose ppm	**Serum Testosterone (ng/g)**

2-Week Treatment	
0	0.76
25	0.83
100	0.54
500	0.90

4-Week Treatment	
0	0.67
25	1.00
100	1.19
500	0.93

8-Week Treatment	
0	0.58
25	0.67
100	0.77
500	0.70

Interestingly, there was no effect on AGD within the two-generation study on sulfoxaflor, which is also consistent with a dopamine agonist/enhancer MoA as maternal prolactin levels during gestation are sufficient to abrogate any prolactin decrease effect in perinatal male rats (*Ben-Jonathan and Hnasko, 2001*).

Key Event #5: Increased Serum LH Levels

In response to transient decreases in testosterone and alteration of LHR gene expression, there is an expected increase in serum LH. With respect to sulfoxaflor, there was an apparent dose-dependent increase in serum LH levels at the 4-week time point in F344/DuCrl rats (table 6.5.4.5-9 and figure 6.5.4.5-5), consistent with the timing of decreased prolactin levels, which were observed in the LCT MoA study (*Rasoulpour et al., 2010a*).

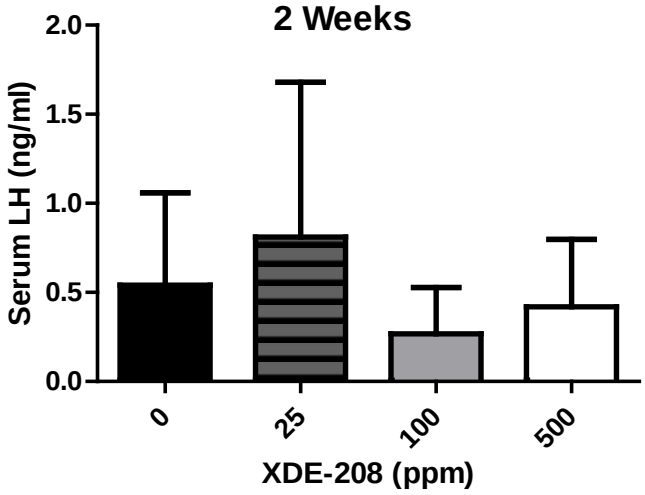
Terminal blood samples were also collected from this LCT MoA study; however, as LH is a pulsitile and stress affected hormone, levels across all groups were induced ~8-10-fold higher than in-life bleeds in response to carbon dioxide euthanasia-associated stress – the complete data can be seen in table 6.5.4.1-4 from section B.6.5.4.1.

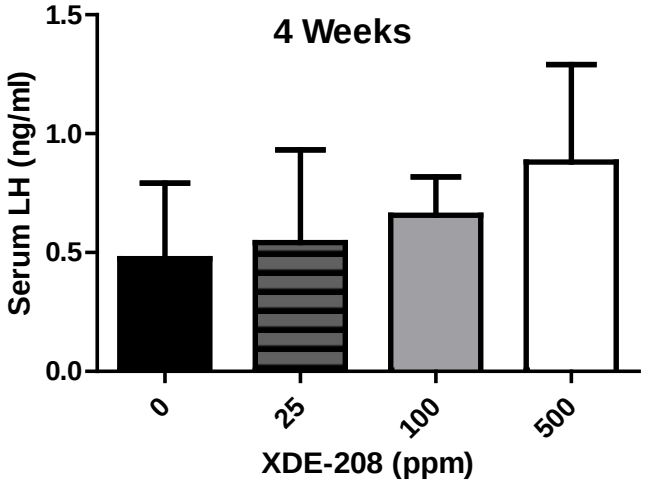
Table 4.10.3.1.Study 11.9 Table 6.5.4.5-9. Sulfoxaflor: F344/DuCrl Rat Serum LH Levels

Dose ppm	Serum LH (ng/ml)
2-Week Treatment	
0	0.54
25	0.81
100	0.27
500	0.42
4-Week Treatment	
0	0.47
25	0.54

100	0.66
500	**0.88**

8-Week Treatment	
0	0.89
25	1.04
100	0.69
500	1.08
Bold type indicates treatment-related effect.	


2 Weeks
Serum LH (ng/ml)
2.0
1.5
1.0
0.5
0.0
0
25
100
500
XDE-208 (ppm)


4 Weeks
Serum LH (ng/ml)
1.5
1.0
0.5
0.0
0
25
100
500
XDE-208 (ppm)

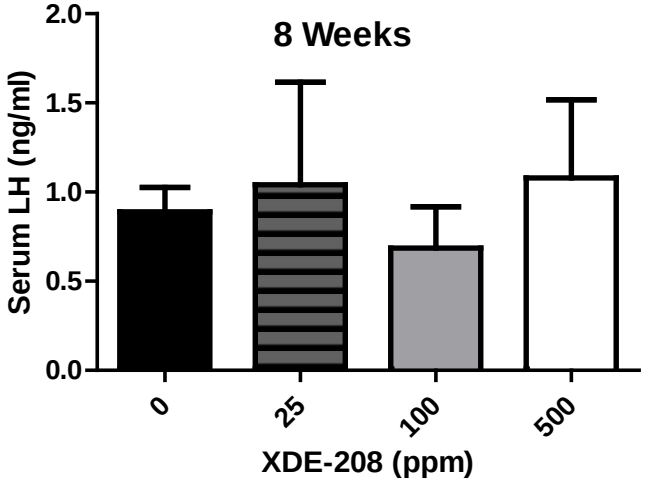

8 Weeks
Serum LH (ng/ml)
2.0
1.5
1.0
0.5
0.0
0
25
100
500
XDE-208 (ppm)

Figure 4.10.3.1.Study 11.5 (DAR Figure 6.5.4.5-5.) Sulfoxaflor: F344/DuCrl Rat Serum LH Levels (Mean ± S.D.)

As outlined in table 6.5.4.5-10, there was no effect of treatment on F344/DuCrl rat hormone levels at the 2- or 8-week time points; however, at 4-weeks there was approximately a ~1.9-fold dose-dependent increase in LH levels concomitant with a ~1.7-fold dose-dependent decrease in prolactin levels.

Table 4.10.3.1.Study 11.10 (DAR Table 6.5.4.5-10.) Sulfoxaflor Key Event #5: Temporal and Dose Response for Serum LH Levels

Temporal →

Dose ↓

Dose ppm	2 weeks	4 weeks	8 weeks
0	100	100	100
25	150	114	116
100	50	140	77
500	78	**187**	121

Data are percentage of control values. **Bold** indicates treatment-related.

Due to the persistent compensatory nature of the HPG axis, coupled with the fact that chronic (i.e., two years) sulfoxaflor exposure was required for marginal effect on PGC incidence in F344/DuCrl rats, it is not surprising that the changes observed in the hormone data are temporal in nature.

Key Event #6: Resetting of the HPG Axis Leading to Increased Serum Testosterone Levels

The rat preputial gland is testosterone dependent for both its proliferation and differentiation (*Miyake et al., 1994; Ponmanickam et al., 2010*). While *Miyake et al.,* (1994) make the point that androgen receptor mRNA is most abundant in the mid-differentiation sebocytes, rather than the less differentiated and more proliferative precursor cells, it is clear from several studies that testosterone provides a key proliferative signal to the rat preputial gland (*Freinkel, 1963; Ponmanickam et al., 2010*). Data to support an increase in serum testosterone due to resetting of the HPG axis mostly come from the peer-reviewed literature with other dopamine agonists/enhancers.

In the LCT MoA study (*Rasoulpour et al., 2011*), 500ppm sulfoxaflor was observed to produce a non-statistically significant increase in serum testosterone at all time points examined (2, 4, and 8 weeks). While the data suggesting elevated serum testosterone after sulfoxaflor exposure are not particularly strong, they are consistent with a functional resetting of the hypothalamic-pituitary-gonadal axis feedback loop to a higher level of activity. That sulfoxaflor can alter the function of

the HPG axis is supported by the two-generation reproductive toxicity study that identified a delay in preputial separation during developmental exposure in the rat. Other dopamine enhancers, such as mesulergine, oxolinic acid, and L-dopa, induced sub-chronic timeframe increases in serum testosterone levels. Oxolinic acid, a quinolone antibiotic that is a dopamine reuptake inhibitor, given to Wistar rats also tended to produce elevated serum testosterone levels during chronic exposure (*Yamada et al., 1994a; Yamada et al., 1994b; Yamada et al., 1995a*). In MoA experiments with the dopaminergic pharmaceutical agent mesulergine, serum testosterone levels were similar to controls at 2-weeks of treatment, slightly lower than controls at 4 weeks, returned to baseline by 10 weeks, and were elevated at 13 weeks (*Prentice et al., 1992*). Finally, L-dopa also produces a trend toward elevated serum testosterone levels in rats (*Yamada et al., 1995b*).

While the resetting of the hypothalamic-pituitary-gonadal feedback loop to a higher level of activity characterised by an elevated serum luteinizing hormone level is common to the different modes of action for rat LCT induction by non-genotoxic chemicals (androgen receptor antagonism, oestrogen receptor agonism/antagonism, 5α-reductase inhibition, aromatase inhibition, reduced testosterone biosynthesis, increased testosterone biliary elimination, GnRH (LHRH) agonism, and dopamine agonism/enhancement), only with the androgen receptor antagonism and dopamine agonism/enhancement modes of action is there an apparent concomitant increase in serum testosterone (*Cook et al., 1999*). In the case of androgen receptor antagonists, the elevated serum testosterone levels are produced to over-ride the inhibition of signaling through the androgen receptor in the hormonal feedback loop. In the case of dopamine agonists, the resulting lower serum prolactin levels and altered Leydig cell luteinizing hormone receptor levels may create a Leydig cell milieu that promotes slightly elevated serum testosterone levels with long-term exposure.

Key Event #7: Promotion of Preputial Gland Tumors

In a two-year rat carcinogenicity study, male F344/DuCrl 344 rats given 500ppm sulfoxaflor had a marginal, not statistically significant, increased incidence in preputial gland carcinoma (PGC) (*Stebbins et al., 2010*). The incidence of PGCs within this study was 5/50, 7/50, 7/50, and 10/50 in males given 0 (controls), 25, 100, or 500ppm, respectively (table 6.5.4.5-11). The marginal increase in high-dose males was conservatively interpreted to be treatment related because the incidence was slightly higher than the historical control range of 0 to 6 PGCs in the four most recent previously conducted F344/DuCrl dietary carcinogenicity studies at the same laboratory. The study where six PGCs occurred was conducted contemporaneously with the sulfoxaflor carcinogenicity study. For sulfoxaflor, the no-observed-effect level for PGCs was the intermediate dose of 100ppm, since the incidence of seven PGCs at 25 and 100ppm was interpreted to be comparable to the historical control range of PGCs.

Table 4.10.3.1.Study 11.11 (DAR Table 6.5.4.5-11.) Preputial Gland Carcinoma					
Sex	**Males**				
Dose (ppm)	**Historical**	**0**	**25**	**100**	**500**

	Control[a]				
Preputial gland gross examination	50	50	50	50	50
Preputial lesion incidence (animals)		8	8	7	**10**
Carcinoma; with or without metastasis	0-6	5	7	7	**10**
Incidence (?)	12%	10%	14%	14%	20%

Bold type indicates treatment-related effect. Histopathological examination of the preputial gland was conducted only when the presence of a gross lesion such as a mass or nodule was observed upon macroscopic examination of the urogenital area containing this gland. See also table 6.5.1.1-13.

Summary of Sulfoxaflor Preputial Gland Carcinoma MoA

The proposed MoA for sulfoxaflor-induced F344/DuCrl 344 rat preputial gland carcinoma promotion is through dopamine enhancement mediated by agonism of the molecule on neuroendocrine dopaminergic nAChRs within the median eminence of the brain in the rat. The relevant end points for this MoA are summarised on table 6.5.4.5-12. This analysis is based on the mechanistic and standard, repeat-dose toxicity studies in rats administered sulfoxaflor.

Table 4.10.3.1.Study 11.12 (DAR Table 6.5.4.5-12.) Sulfoxaflor: Temporality and dose response for MoA key events related to male F344/DuCrl rat preputial gland carcinoma

Temporal →

Dose ↓

Dose (ppm)	**Key Event 1**	**Key Event 2**	**Key Event 3**	**Key Event 4**	**Key Event 5**	**Key Event 6**	**Key Event 7**
	Increased dopamine release via nAChR agonism	Decreased serum prolactin levels	Downreg of LHR gene expression in Leydig cells	Transient decreased serum testosterone levels	Increased serum LH levels	Reset of HPG axis / increased serum testosterone	Promotion of preputial gland tumors

25		-	-	-	-		-
100		-	-	-	-		-
400				+*			
500	+	+	+	-	+	**	+
+ indicates effect present, - indicates effect absent, blank cell indicates no data; * indicates indirect data from delay in balanopreputial separation data; **indicates no direct data, but supportive evidence in the literature.							

(D) Proposed timeline for sulfoxaflor-induced rat preputial gland carcinoma:

In the aging rat, and in particular in aging F344/DuCrl rats (*Amador et al., 1985*), the physiology of the hypothalamic-pituitary-gonadal axis undergoes a progressive change. The axis becomes progressively less responsive, and serum luteinizing hormone levels fall. It is also known that as LCTs markedly increase in size, which occurs late in life, they progressively lose the ability to synthesise testosterone and instead secrete increasing amounts of progesterone, a precursor of testosterone synthesis (*Amador et al., 1985*).

These changes in serum hormones over a lifetime of exposure to sulfoxaflor in the F344/DuCrl rat are summarised in figure 6.5.4.5-6. Shown are the postulated serum levels of progesterone (top lines, yellow), luteinizing hormone (middle lines, red), and testosterone (bottom lines, blue) over the duration (log scale) of sulfoxaflor exposure compared to controls in the F344/DuCrl rat 2-year carcinogenicity study.

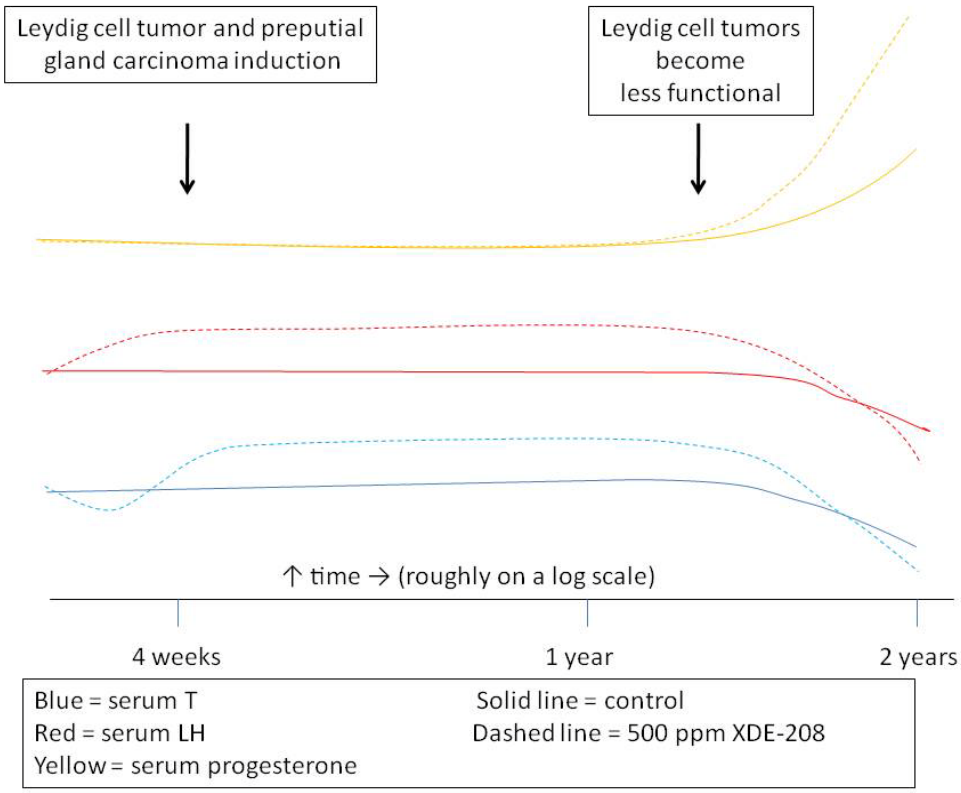


Figure 4.10.3.1.Study 11.6 (DAR Figure 6.5.4.5-6): Postulated changes in serum hormones over a lifetime of exposure to sulfoxaflor in the F344/DuCrl rat.

In support of the LCT MoA for sulfoxaflor due to dopamine enhancement, there were slight increases in serum luteinizing hormone and testosterone levels during the majority of the rat lifespan. Preputial gland carcinoma development resulted from life-long elevated serum testosterone levels, and likely progressed through a classical hyperplasia to adenoma to carcinoma pathogenetic sequence. The LCT MoA for sulfoxaflor is one of the only MoAs that can cause sustained increases in serum testosterone with a resultant enhancement of PGCs.

Common to all LCT MoAs are the anti-androgenic secondary effects at the end of life. At two years of age, levels of these hormones fall, associated with aging of the hypothalamic-pituitary-gonadal axis and the progressive enlargement of LCTs and their loss of functionality. The fall in serum luteinizing hormone and testosterone is accompanied by an increase in progesterone secretion by the enlarging LCTs. The enlarging LCTs compromise the normal functioning of Leydig cells and the seminiferous tubules resulting in seminiferous tubule atrophy, decreased spermatogenesis, fewer spermatic elements in the epididymides, and decreased secretions in the secondary sex organs.

(E) Alternative MoA analysis:

A detailed evaluation of the alternative MoAs for Leydig cell tumors are presented within the

Leydig cell tumour MoA/ human relevance framework (HRF) document (section B.6.5.4.4; *Rasoulpour et al., 2011*).

(F) Sulfoxaflor preputial gland carcinoma human relevance framework

Question 1. Is the weight of evidence sufficient to establish the MoA in animals?

The answer is yes. The MoA for sulfoxaflor-induced F344/DuCrl rat preputial gland tumours is compatible with that described for dopamine agonists/enhancer-induced Leydig cell tumours (*Cook et al., 1999*). The available data for sulfoxaflor presented in this MoA/HRF provide evidence supporting MoA #9 in the form of increased dopamine release from the hypothalamus, decreased circulating Prl levels, with increased LH, along with decreased testis LHR gene expression. Chronically increased testosterone levels are likely (Other dopamine enhancers, such as mesulergine, oxolinic acid, and L-dopa, induced increases in serum testosterone levels (*Prentice et al., 1992, Yamada et al., 1994a, Yamada et al., 1995b*), though direct data for sulfoxaflor does not exist. This MoA operates through sulfoxaflor-mediated enhancement of dopamine release, most likely through agonism of central nicotinic acetylcholine receptors, which play a key regulatory role in dopamine release from dopaminergic neurons in the brain (*Maskos, 2010*). As mentioned previously, sulfoxaflor is an agonist to the foetal rat muscle nAChR and the insect neuronal nAChR is the target of the insecticidal mechanism for sulfoxaflor. Based on these data, it is plausible that the PGC promotion seen in the rat chronic/carcinogenicity study was through subtle, but chronic, enhancement of dopamine release, and subsequent inhibition of prolactin release from the pituitary gland, ultimately leading to an increase in serum testosterone over the course of the carcinogenicity study. This is a hormonally-mediated, threshold based, nonlinear MoA.

Question 2. Can human relevance of the MoA be reasonably excluded based on fundamental qualitative differences in key events between experimental animals and humans?

The answer is yes. As previously discussed, this MoA/HRF was designed to evaluate the MoA for the slightly increased incidence of PGC observed in the sulfoxaflor 2-year rat oncogenicity study at 500ppm. There are quantitative and qualitative interspecies differences of Leydig cell response to hormonal stimuli that are well understood (*Cook et al., 1999*). Rat Leydig cells contain >10-fold more LH receptors than humans, which confers greater sensitivity to slight changes in LH levels (*Huhtaniemi, 1983; Katzung, 1995*). In addition to this quantitative difference, rat, but not human, Leydig cells have PrlRs on their surface (*Clayton and Huhtaniemi, 1982; Cook et al., 1999*). Stimulation of rat Leydig cells through PrlR are a rat-specific mechanism: human Leydig cells do not have functional prolactin receptors and hence the sequence of events beyond this step cannot occur in humans.

A concordance analysis of the key events for a dopamine agonism/enhancement MoA is presented in table 6.5.4.5-13.

Table 4.10.3.1.Study 11.13 (DAR Table 6.5.4.5-13.) Concordance of Key Events for a Dopamine Agonism/Enhancement PGC MoA in Rodents and Humans		
Key Event	**Evidence in Rodents**	**Evidence in Humans**
Increased dopamine release via nAChR agonism	Yes	Yes
Decreased serum prolactin levels	Yes	Yes
Reduced stimulation of prolactin receptors on Leydig cells resulting in reduced LH receptor density on Leydig cells	Yes	No; unlike rat Leydig cells, human Leydig cells do not possess a prolactin receptor and there is no evidence of human Leydig cell tumours from dopamine agonist treatments for hyperprolactinemia or Parkinson's disease
Downreg of LHR gene expression in Leydig cells	Yes	No; none reported
Transient decreased serum testosterone levels	Yes	No; none reported
Reset of HPG axis / increased serum testosterone	Yes	No; based on epidemiological data
Promotion of preputial gland tumors		

Question 3. Can human relevance of the MoA be reasonably excluded based on quantitative differences in either kinetic or dynamic factors between experimental animals and humans?

As human relevance of the experimental animal MoA can be reasonably excluded on the basis of qualitative differences in key events (Question 2); a quantitative assessment of kinetic or

dynamic factors is not necessary. However, as described in the background section of this document, there are significant differences in the background incidence of LCT across species and strains (the shared MoA with PGC), with F344/DuCrl rats being the most and humans being the least, sensitive. The biological basis for these differences in susceptibilities is described in detail within the background section of this report and includes both qualitative and quantitative differences in the underlying biology between rat and human Leydig cells.

(G) Weight of evidence for no relevance of sulfoxaflor preputial gland carcinoma for human health risk assessment:

Overall, the weight of evidence (WoE) supports no relevance of preputial gland carcinomas for human health risk assessment because:

- The MoA for sulfoxaflor-induced preputial gland carcinoma is not relevant to humans
- Sulfoxaflor has no indication of genotoxicity from *in vitro* and *in vivo* assays for mutagenicity or clastogenicity
- Humans do not have an anatomic equivalent to rodent preputial glands
- There were no effects in the female rat correlate to the preputial gland (clitoral gland)
- Even at far higher doses, there were no effects in CD-1 mouse preputial glands, clitoral glands, or other sebaceous glands (skin, Zymbal's gland)
- There were no effects in other sebaceous glands (skin, Zymbal's gland) in male or female F344/DuCrl rats.

The MoA for sulfoxaflor-induced PGC is not relevant to humans.

As described in the previous MoA/HRF sections of this document, the MoA for sulfoxaflor-induced PGC is proposed as dopamine enhancement, which is considered not relevant to humans.

Sulfoxaflor is not genotoxic.

Sulfoxaflor was clearly negative in the battery of *in vitro* and *in vivo* genotoxicity assays for mutagenicity and clastogenicity. These included the bacterial reverse mutation (Ames) test (*Mecchi, 2007*), *in vitro* mammalian chromosome aberration (RLCAT) test (*Schisler et al., 2007a*), the *in vitro* mammalian cell gene mutation (CHO/HGPRT) test (*Schisler et al., 2007b*), and the mammalian erythrocyte micronucleus (MNT) test (*LeBaron and Schisler, 2009*).

Humans do not have an anatomical equivalent to rodent preputial glands

The rat preputial gland is a modified sebaceous gland that has a role in pheromone secretion and sexual behavior (*Ponmanickam et al., 2010*). There is no anatomical equivalent of the rat preputial gland in the human (*Monro and Mordenti, 1995*).

No effect in other sebaceous tissues

It should be noted that in the sulfoxaflor two-year carcinogenicity study, there were no treatment-related effects in any of the other sebaceous tissues of the body. These include the clitoral gland of female rats, the perifollicular sebaceous glands of the skin, and the auditory sebaceous (Zymbal's) glands. The lack of proliferative effects in these sebaceous sites is consistent with the testosterone-driven mode of action for enhancement of PGC development in high-dose males. Female rats would not have an sulfoxaflor dependent increase in testosterone, due to their lack of Leydig cells, so there would be no stimulus for an enhanced incidence of clitoral gland proliferative lesions. The cutaneous sebaceous glands and the auditory sebaceous gland would not be expected to be responsive to testosterone as compared to the preputial gland.

No metabolism of sulfoxaflor

A pharmacokinetic and metabolism study (*Hansen, et al., 2009*) of sulfoxaflor in F344/DuCrl rats have shown that the compound was rapidly and highly absorbed, poorly metabolised and readily eliminated in urine from the rat, with low tissue residues. Therefore, there it is highly unlikely that a metabolite of sulfoxaflor could be responsible for the higher incidence of PGCs in high-dose males.

Annex II: MoA for toxicity to reproduction

Study 1: Rat cross-fostering study (DAR B.6.6.12.)

The purpose of this study was to determine whether the previously observed decreased survival of pups born to sulfoxaflor-treated dams resulted from *in utero* and/or lactational exposure. As part of this study, effects on general toxicity, toxicokinetic analysis of blood and milk, reproductive function and prenatal/early neonatal growth and survival were assessed.

Groups of female Crl:CD(SD) rats were fed diets supplying 0 (control) or 1000 ppm sulfoxaflor for two weeks prior to mating through weaning on lactation day (LD) 21. As the control and treated females mated, they were subdivided into Foster dams and Donor dams. Cesarean-section was performed on gestation day (GD) 21 Donor dams, at which time, one or more batches of two of their offspring/sex were immediately cross-fostered to a Foster dam(s) that had their own litter removed that day (i.e., on LD 0). After cross-fostering was complete, each control and sulfoxaflor-treated Foster dam had mixed litters comprised of two pups/sex that originated from control Donor dams (five litters) and two pups/sex that originated from sulfoxaflor-treated Donor dams (eight litters). This design controlled for litter of origin effects, and enabled comparison of the survival of pups exposed to sulfoxaflor during gestation alone or during lactation alone with unexposed control pups and pups exposed during both gestation and lactation.

Dams given 1000 ppm sulfoxaflor had treatment-related effects on body weight, body weight gain, and feed consumption consistent with effects seen at this dose level in the previous reproduction/developmental toxicity screening study. Time weighted average doses for treated animals were 81.2, 74.5, and 59.5 mg/kg/day in the pre-mating, gestation, and lactation periods, respectively. These corresponded to maternal sulfoxaflor blood concentrations of 23.0-29.3 µg/g plasma on GD 21 and 19.6-25.0 µg/g plasma on LD 0. The average measured plasma concentration of sulfoxaflor of male/female pups on GD 21 and LD 0 from these dams was 24.8/24.8 and 25.3/25.9 µg sulfoxaflor/g plasma, respectively. Thus, foetal and pup plasma levels of sulfoxaflor were very similar to one another, and very similar to dam plasma levels. The measured milk concentration from the same dams on LD 0 were approximately half the corresponding plasma levels and ranged from 12.3-14.0 µg sulfoxaflor/g milk (mean = 13.3 µg/g)

All offspring from dams exposed to 1000 ppm sulfoxaflor prior to birth died by postnatal day (PND) 4, irrespective of whether they were cross-fostered to control- or treated-foster dams (see results table below). Consistent with reduced viability, some offspring were cold to the touch, had bluish skin, autolysed and cannibalised, and stomach void of milk. Conversely, there was no effect on neonatal survival for pups exposed to sulfoxaflor only after birth. Furthermore, PND 1 pup body weights were significantly decreased in prenatally exposed offspring. In conclusion, these data demonstrate that the effect of sulfoxaflor on pup survival was due to *in utero*, not lactational, exposure.

Table 4.11.3.1.Study 1.1 (DAR Table Cross Foster or Treated Foster Dams Results)

Foster Dams	Donor Pups	Hypotheses for Pup Survival	Outcome
Control	Control *in utero*	**No effect expected** (negative control)	No effect
	Treated *in utero*	If pups die, effect comes from treated **pups** (i.e., ***in utero* effect**)	All pups died by PND4
XDE-208 1000 ppm	Control *in utero*	If pups die, effect comes from treated **dams** (i.e., **lactational effect**)	No effect
	Treated *in utero*	**Pup death expected** (positive control)	All pups died by PND4

This study non-guideline study is acceptable.

Report: XDE-208: A Dietary Reproductive Toxicity Cross-Fostering Study in Crl:CD(SD) Rats
Author: R. J. Rasoulpour, Ph., Zablotny, C.L. (2010d)
Date of Report: 01 July, 2010
Report Identity: Study ID: 081122

Testing Facility: Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan, 48674.
GLP Yes
Test Substance: XDE-208 (95.6% (wt/wt); as two diastereomers in 48.4/47.4% ratio.
Batch: E2162-34 TSN003725-0001
Guidelines: Non-guideline
Deviations: Not applicable
Acceptable: Yes

Materials and Methods
In a non-guideline cross-fostering dietary developmental toxicity study groups of 16 female Crl:CD(SD) rats were administered sulfoxaflor (purity 95.6% wt/wt, Lot # E2162-34, TSN003725-001) at dietary concentrations of 0 or 1000 ppm. This study utilized a single dose level of 1000 ppm because this dose produced a significant decrease in pup survival in the preceding OECD 421 study. The purpose of this study was to determine whether the previously observed decreased survival of pups born to sulfoxaflor-treated dams resulted from in utero and/or lactational exposure. As part of this study, effects on general toxicity, toxicokinetic analysis of blood and milk, reproductive function and prenatal/early neonatal growth and survival were assessed.

Groups of female Crl:CD(SD) rats were fed diets supplying 0 (control) or 1000 ppm sulfoxaflor for two weeks prior to mating through weaning on lactation day (LD) 21. As the control and treated females mated, they were subdivided into Foster dams and Donor dams. Cesarean-section was performed on gestation day (GD) 21 Donor dams, at which time, one or more batches of two of their offspring/sex were immediately cross-fostered to a Foster dam(s) that had their own litter removed that day (i.e., on LD 0). After cross-fostering was complete, each control and sulfoxaflor-treated Foster dam had mixed litters comprised of two pups/sex that

originated from control Donor dams (five litters) and two pups/sex that originated from sulfoxaflor-treated Donor dams (eight litters). This design controlled for litter of origin effects, and enabled comparison of the survival of pups exposed to sulfoxaflor during gestation alone or during lactation alone with unexposed control pups and pups exposed during both gestation and lactation.

Table 4.11.3.1.Study 1.2 (DAR Table B.6.6.12.1-1): Summary of Key Study Parameters and Study Schedule

Study Events and Parameters	No. Animals[1]	Timing
Cage-side examinations	All	At least twice daily
Clinical observations-adult females	All	Weekly; GD 0, 7, 14, and 20; LD 1, 4, 7, 14, 21
Clinical observations-adult males	All	Pre-study
Body weights – adult females	All	Weekly; GD 0, 7, 14, 20; LD 1, 4, 7, 14, 21; at termination
Body weights – adult males	All	Pre-study
Feed consumption – adult females	All	Weekly during pre-breeding period; GD 0, 7, 14, 20; LD 1, 4 , 7, 11, 14, 17, 19, 21
Cesarean-Section	Donor dams	GD 21
Donor Dam Kinetics (blood)	Donor dams	GD 21
Donor Foetus Kinetics (blood)	1 Non-cross-fostered foetus/sex/litter (first four littered)	GD 21
Dam Kinetics (blood and milk)	4 dams/dose	LD 0
Pup Kinetics (blood)	1/sex/from row above	PND 0
Litter clinical observations	All	LD 0, 1, 4, 7, 14, 21
No. of live & dead pups	All	PND 0, 1, 4, 7, 14, 21
Pup sex & body weight	All	PND 1, 4, 7, 14, 21
Weaning & Termination of pups	All	PND 21

[1]Adults unless specified otherwise; GD = gestation day, LD = lactation day, PND = postnatal day, N/A = not applicable

Table 4.11.3.1.Study 1.3 (DAR Table B.6.6.12.1-2): Animal Assignment

	No. of Adult Females[1]	No. of Adult Males[2]	Sulfoxflor	Cross-Foster
Foster Control	16	8	0 PPM	Natural delivery, pups removed
Foster sulfoxaflor	16	8	1000 PPM	Natural delivery, pups removed
Donor Control	16	8	0 PPM	GD 21, Cesarean-Section
Donor sulfoxaflor	16	8	1000 PPM	GD 21, Cesarean-section
Total no. adults/sex	64	32		

Results:

Test material: Analysis of the 1000 ppm diets indicated that the test material was homogeneously distributed based on relative standard deviations of 1.0 and 1.7%. A previously conducted stability study showed sulfoxaflor to be stable for at least 65 days in rodent feed at concentrations ranging from 0.0005 to 10%. Test diets for the current study were prepared and

used within these stability limits.
Analyses of the 1000 ppm diets, plus control and premix revealed concentrations ranging from 95.8 to 97.3% of targeted concentrations.

In life observations: Parental
Mortality and clinical signs: All parental animals survived until termination.
Body weights: Females given 1000 ppm sulfoxaflor had treatment-related effects on body weight and body weight gain (Tables B.6.6.12.1-3) as demonstrated in the previous reproduction/developmental screening study. These consisted of a 20% decrease in GD 0-7 body weight gain and a mean 5.3 g loss in LD 1-4 body weight compared to an 8.3 g gain in controls during that period. Thus, sulfoxaflor-treated dams weighed 13% less than controls on LD 4 and 7. Body weight/body weight gain was similar between animals given control and 1000 ppm sulfoxaflor at other intervals throughout the study.

Table 4.11.3.1.Study 1.4 (DAR Table B.6.6.12.2-3): Mean body weights/weight gains of pregnant dams.

GESTATION			LACTATION		
DAY OF GESTATION	0 PPM[A]	1000 PPM[B]	DAY OF LACTATION	0 PPM[C]	1000 PPM[D]
0	243.4 ± 13.7	234.9 ± 11.6	1	299.1± 19.7	272.0 ± 11.6
7	281.4 ± 16.4	265.3 ± 15.3	4	307.4 ± 19.8	266.7 ± 9.9
14	313.1 ± 19.5	299.3 ± 18.1	7	320.8 ± 22.7	278.0 ± 8.7
21	383.4 ± 24.9	370.2 ± 22.4	14	335.5 ± 23.0	300.5 ± 12.3
			21	327.8 ± 23.1	296.3 ± 15.4
MEAN (±SD) BODY WEIGHT GAINS (G) OF PREGNANT FEMALES					
0-7	38.1 ± 6.6	30.4 ± 7.0	1-4	8.3 ± 6.4	-5.3 ± 4.5
7-14	31.6 ± 7.2	34.0 ± 6.6	4-7	13.4 ± 5.1	11.3 ± 3.5
14-20	70.3 ± 14.7	70.9 ± 9.6	7-14	14.7 ± 4.0	22.4 ± 4.9
0-20	140.0 ± 20.3	135.3 ± 14.8	14-21	-7.7 ± 5.3	-4.1 ± 7.7
			1-21	28.7 ± 11.5	24.3 ± 8.4

a N=29; b N=25; c N=5; d N=8

Food consumption/substance intake: As seen in the previous reproduction/developmental screening study, there were treatment-related effects on feed consumption for animals given 1000 ppm sulfoxaflor. These were particularly evident during the first week of the study (13.8% lower than controls) and during the first four days of lactation (30.3% lower than controls) (Table B.6.6.12.2-4). Test Substance Index Females were given diets containing 0 and 1000 ppm sulfoxaflor. These values corresponded to time weighted average doses of 0 and 81.2 mg/kg/day in the pre-mating phase, 0 and 74.5 mg/kg/day during gestation and 0 and 59.5 mg/kg/day during lactation.

Cross-foster: Initially, isoflurane was used to anesthetize donor dams during Cesarean section. Offspring from these 0 and 1000 ppm donor dams were cross-fostered onto 0 and 1000 ppm foster dams. It was discovered that most donor pups, including control 0/0 (0 ppm in utero / 0 ppm lactation) were dying within hours of cross-fostering due to exposure to isoflurane anesthesia during Cesarean section. Therefore the anesthetic agent was switched to carbon dioxide. Due to this confounding factor, animals in all groups exposed to isoflurane were excluded from analysis but are presented on the individual summary tables for completeness. This resulted in a sample size of five litters cross-fostered to 0 ppm dams and eight cross-fostered to 1000 ppm sulfoxaflor dams.

Toxicokinetics: There was no sulfoxaflor found in plasma obtained from dams or pups of the control group on GD 21. The measured plasma concentration in dams treated with 1000 ppm sulfoxaflor ranged from 23.0-29.3 µg sulfoxaflor /g plasma on GD 21 (mean = 22.4) and 19.6-25.0 µg sulfoxaflor /g plasma on LD 0 (mean = 27.0). The average measured plasma concentration of sulfoxaflor of male/female pups on GD 21 and LD 0 from these dams was 24.8/24.8 and 25.3/25.9 µg sulfoxaflor /g plasma, respectively. Thus, foetal and pup plasma levels of sulfoxaflor were very similar to one another, and very similar to dam plasma levels. The measured milk concentration from the same dams on LD 0 were approximately half the corresponding plasma levels and ranged from 12.3-14.0 µg sulfoxaflor/g milk (mean = 13.3

µg/g).

Sample collection:	Dams	Male P	Female P
GD 21 (µg/g plasma)	27.0 ± 10.2	24.8 ± 10.6	24.8 ± 10.4
LD 0 (µg/g plasma)	22.4 ± 10.2	25.3 ± 4.21	25.9 ± 6.94
LD 0 (µg/g milk)	13.3 ± 6.30	N/A	N/A

Observations: Offspring

Viability/clinical signs: Pups observations were summarised in these tables by the lactation (foster) dam without regard to exposure group during gestation. Donor pups from dams treated with 1000 ppm prior to birth had litter observations associated with decreased survival. These observations included pups found dead, cold to the touch, bluish skin, autolysed pups, cannibalised pups, and/or stomach void of milk

Survival: Administration of 1000 ppm sulfoxaflor during gestation and lactation had been previously shown to induce decreased pup survival between PND 0-4. In the current study, all donor pups from dams treated with 1000 ppm sulfoxaflor prior to birth died by PND 4, irrespective of whether they were cross-fostered onto control or treated foster dams. In contrast, there was no treatment-related effect on postnatal survival in donor pups derived from control dams and cross-fostered onto treated foster dams. Taken together these data demonstrate that the early postnatal pup death was due to gestational exposure and not lactational exposure to sulfoxaflor.

Table 4.11.3.1.Study 1.5 (DAR Table B.6.6.12.1-4): Effects on Pup Survival

[a]Dose (ppm):	0/0	1000/0	0/1000	1000/1000
PND 1 Survival (%)	100.0 (20/20)	**15.0** (3/20)	96.9 (31/32)	**40.6** (13/32)
PND 4 Survival (%)	100.0 (20/20)	**0.0** (0/20)	96.9 (31/32)	**0.0** (0/32)

[a] - Dose in gestation/dose in lactation

Bold type indicates the effects judged to be treatment-related.

Litter size: Consistent with the previously described effects on postnatal survival, litter size was markedly decreased in litters with donor pups from dams treated with 1000 ppm sulfoxaflor prior to birth with all pups in this group dead by PND 4. In addition to the effects on pup survival and litter size and consistent with previous studies, there was a treatment-related decrease in pup body weights from dams treated with sulfoxaflor prior to birth.

Table 4.11.3.1a Study 1.6 (DAR Table B.6.6.12.1-5a): Selected Litter Size

	Mean Litter Size			
[C]**Dose (ppm)**	[a]0/0	[a]1000/0	[b]0/1000	[b]1000/1000
#pups Crossfostered /Litter	4.0	4.0	4.0	4.0
PND 1	4.0	**0.6**	3.9	**1.6**
PND 4	4.0	**0.0**	3.9	**0.0**

[a] Dose in gestation/dose in lactation
Bold type indicates the effects judged to be treatment related.
N=5 Litters 0/0 and 1000/0
[b] Only 1 litter with pups remaining on PND 1
N=5 litters 0/1000 and 1000/1000
[C] N=4 litters.

Table 4.11.3.1b Study 1.6 (DAR Table B.6.6.12.1-5b Selected Pup Body Weights)

	Mean pup body weights (female/male)			
Dose (ppm)a	0/0	1000/0	0/1000	1000/1000
PND 0	5.2/5.5	5.1/5.2	5.3/5.7	5.1/5.5
PND1	5.5/6.0	**4.8/4.5#**	5.8/6.3	**4.6/4.8**

only 1 litter on PND1
Bold type indicates the effects judged to be treatment related.
[a] Dose in gestation/dose in lactation

Conclusions

Dams given 1000 ppm sulfoxaflor had treatment-related effects on body weight, body weight gain, and feed consumption consistent with effects seen at this dose level in the previous reproduction/developmental toxicity screening study. Time weighted average doses for treated animals were 81.2, 74.5, and 59.5 mg/kg/day in the pre-mating, gestation, and lactation periods, respectively. These corresponded to maternal sulfoxaflor blood concentrations of 23.0-29.3 µg/g plasma on GD 21 and 19.6-25.0 µg/g plasma on LD 0. The average measured plasma concentration of sulfoxaflor of male/female pups on GD 21 and LD 0 from these dams was 24.8/24.8 and 25.3/25.9 µg sulfoxaflor/g plasma, respectively. Thus, foetal and pup plasma levels of sulfoxaflor were very similar to one another, and very similar to dam plasma levels. The measured milk concentration from the same dams on LD 0 were approximately half the corresponding plasma levels and ranged from 12.3-14.0 µg sulfoxaflor/g milk (mean = 13.3 µg/g)

All offspring from dams exposed to 1000 ppm sulfoxaflor prior to birth died by postnatal day (PND) 4, irrespective of whether they were cross-fostered to control- or treated-foster dams (see results table below). Consistent with reduced viability, some offspring were cold to the touch, had bluish skin, autolysed and cannibalised, and stomach void of milk. Conversely, there was no effect on neonatal survival for pups exposed to sulfoxaflor only after birth. Furthermore, PND 1 pup body weights were significantly decreased in prenatally exposed offspring. In conclusion, these data demonstrate that the effect of sulfoxaflor on pup survival was due to *in utero*, not lactational, exposure.

Study 2: Rabbit neonatal survival study (DAR B.6.6.12.2)

Sulfoxaflor, was offered on a continuous basis in the diet (with 0.5% apple flavoring) to a group of 12 litter-experienced, time mated female New Zealand White [Hra:(NZW)SPF] rabbits from gestation day (GD) 7 through the initiation of parturition (25-26 consecutive days). The target test substance concentration of 750 ppm was achieved (101.5% of target concentration), and reflected a maximum tolerated exposure based on previous studies in this species. Actual test material intake in the 750 ppm group was 29 mg/kg/day during GD 7-28. A concurrent control group of 12 time-mated females received the apple-flavored control diet on a comparable regimen. All diets were formulated according to the specifications for Purina Mills International (PMI) Certified Rabbit LabDiet® 5325 and were provided at 150 g/day ± 5 g/day during the exposure period (GD 7 through initiation of parturition) and at 200 g/day ± 5 g/day during lactation days (LD) 1-4; the control diet was offered to both groups after parturition. The F0 females were approximately 9-13 months of age at the initiation of test substance exposure. All animals were observed twice daily for mortality and moribundity. Clinical observations, body weights, and food consumption were recorded at appropriate intervals. All F0 females were allowed to deliver and rear their offspring to LD 4. All F0 females were necropsied within 24 hours of total litter loss, on LD 4, or on post mating day 37. All surviving F1 offspring received a detailed physical examination on postnatal day (PND) 4 and were then discarded.

With the exception of 1 F0 female in the control and 750 ppm groups euthanized on LD 3 due to total litter loss, all females survived to the scheduled necropsies. No test substance-related maternal macroscopic findings were noted.

Lower mean body weight gains (24.2%) and food consumption (7.3%) were noted in the 750 ppm group during the gestation exposure period compared to the control group. Corresponding incidences of decreased defecation were noted for 3 females in this group. Although mean body weights remained within 2.9% of control group values throughout gestation, the reductions in mean body weight gains and food consumption were attributed to test substance exposure. Mean body weights, body weight gains, and food consumption in the 750 ppm group were similar to the control group during LD 1-4.

No test substance-related effects were observed on the mean number of offspring born, offspring survival, or the general physical condition of the offspring.

Based on these results, an exposure level of 750 ppm, equivalent to 29 mg/kg/day, was considered to be the no observed effect level (NOEL) for neonatal survival when sulfoxaflor was offered continuously in the diet from GD 7 through the initiation of parturition to pregnant New Zealand White rabbits. In contrast to the rat, sulfoxaflor was not developmentally toxic in the rabbit, despite the achievement of similar maternal and foetal systemic concentrations of sulfoxaflor in both species.

Report:	A Study of the Effect of XDE-208 on Neonatal Survival in New Zealand White Rabbits
Author:	Kuhl, A.J.
Date of Report:	04 August, 2009
Report Identity:	Study ID: WIL-410011
Testing Facility:	WIL Research Laboratories, LLC, Ashland, OH, 2009.

GLP	Yes
Test Substance:	XDE-208 (95.6% (wt/wt); as two diastereomers in 48.4/47.4% ratio.
Batch:	E2162-34
Guidelines:	Non-guideline
Deviations:	Not applicable
Acceptable:	Yes

Materials and Methods

Sulfoxaflor was offered on a continuous basis in the diet (with 0.5% apple flavouring) to a group of 12 litter-experienced, time mated female New Zealand White [Hra:(NZW)SPF] rabbits from gestation day (GD) 7 through the initiation of parturition (25-26 consecutive days). The target test substance concentration of 750 ppm was achieved (101.5% of target concentration), and reflected a maximum tolerated exposure based on previous studies in this species. Actual test material intake in the 750 ppm group was 29 mg/kg/day during GD 7-28. A concurrent control group of 12 time-mated females received the apple-flavored control diet on a comparable regimen. All diets were formulated according to the specifications for Purina Mills International (PMI) Certified Rabbit LabDiet® 5325 and were provided at 150 g/day ± 5 g/day during the exposure period (GD 7 through initiation of parturition) and at 200 g/day ± 5 g/day during lactation days (LD) 1-4; the control diet was offered to both groups after parturition. The F_0 females were approximately 9-13 months of age at the initiation of test substance exposure. All animals were observed twice daily for mortality and moribundity. Clinical observations, body weights, and food consumption were recorded at appropriate intervals. All F_0 females were allowed to deliver and rear their offspring to LD 4. All F_0 females were necropsied within 24 hours of total litter loss, on LD 4, or on post mating day 37. All surviving F_1 offspring received a detailed physical examination on postnatal day (PND) 4 and were then discarded.

Results

Maternal observations

Mortality and clinical signs: With the exception of 1 female each in the control and 750 ppm groups that were euthanised on LD 3 due to total litter loss, all females survived to the scheduled necropsy on LD 4 or post-mating day 37. Test substance related clinical findings were limited to decreased defecation for 3 females (2 to 6 incidences each) in the 750 ppm group during GD 9-15, corresponding to slight reductions in food consumption for these individual animals. Other clinical findings noted in the 750 ppm group, including hair loss and scabbing on various body surfaces, soft stool, and small faeces, occurred infrequently, at similar frequencies in the control group, and/or in a manner that was not exposure-related.

Body weights and food consumption: Slight effects on mean maternal body weight gains or body weight losses were noted generally throughout the exposure period in the 750 ppm group, resulting in an overall lower mean body weight gain during GD 7-28 compared to the control group; differences did not achieve statistical significance. Although mean body weights in the 750 ppm group remained within 2.9% of control group values throughout gestation, the reduction in mean body weight gain during GD 7-28 was attributed to test substance exposure, but of limited toxicological relevance.

Mean maternal body weight and body weight gain in the 750 ppm group were similar to the control group during LD 1-4. Differences from the control group were slight and not statistically significant.

Mean maternal food consumption, evaluated as g/animal/day and g/kg/day, in the 750 ppm group (132 g/animal/day to 145 g/animal/day) was slightly lower (not statistically significant) during the exposure period compared to the control group animals, which generally consumed all 150 g/day offered. These differences corresponded to lower mean body weight gains in this group and were attributed to test substance exposure. All animals in the control and 750 ppm groups consumed all 200 g/day offered during LD 1-4.

Gestation Length and Parturition: No test substance-related effects were noted on mean gestation length or the process of parturition at 750 ppm. Mean gestation length in the test substance-exposed group (31.3 days) was similar to and not statistically different from the control group value (31.5 days). No signs of dystocia were noted.

Gross Pathology: No exposure-related internal findings were noted. Macroscopic findings observed in the 750 ppm group were limited to an accessory spleen and white areas on the kidneys and occurred in single females.

Offspring observations

PND 0 Litter Data and Postnatal Survival: The mean number of offspring born, live litter size, and postnatal survival between birth and PND 0 (relative to number born), PND 0-1, 1-4, and from birth to PND 4 were unaffected by the F_0 maternal test substance exposure. Differences between the control group and the 750 ppm group were slight and not statistically significant. Mean postnatal survival from birth to PND 4 in the 750 ppm group was 71.6% per litter compared to 72.7% per litter in the concurrent control group and a range of 68.4% to 87.7% per litter in the historical control data. One female in the 750 ppm group had a total litter loss on LD 3. However, because a total litter loss was noted on this same day in the control group and total litter loss was noted for 4 females in the WIL historical control data (57 litters evaluated) during PND 0-4, the single total litter loss in the 750 ppm was not attributed to maternal test substance exposure.

General Physical Condition: The general physical condition of all F1 offspring in this study were unaffected by F0 maternal test substance exposure. Offspring (litters) that were found dead numbered 34(7) and 35(9) in the control and 750 ppm groups, respectively.

Table 4.11.3.1.Study 2.1 (DAR Table B.6.6.12.2-1.): Mean (±SD) Dietary study of sulfoxaflor effects on neonatal survival – Summary of PND 0 -4 Litter Data (% per litter)

	N	Number Born	Average Live litter size (PND 0)	Postnatal survival PND 0 (relative to number born)	Postnatal survival PND 0 - 1	Postnatal survival PND 1 - 4	Postnatal survival Birth - PND 4	Found Dead (pups(litters))
Control (0ppm)	11	10.9±2.88	10.8±2.82	99.3±2.32	97.8±5.0	74.2±37.46	72.7±36.99	34(7)
750 ppm	12	10.6±2.27	9.8±2.86	93.8±19.18	99.2±2.89	78.4±29.29	71.6±30.81	35(9)

Conclusion

Lower mean maternal body weight gains and food consumption and corresponding clinical findings of decreased defecation were noted in the 750 ppm group which were attributed to test substance exposure, but are not considered toxicologically significant. No test substance related effects were noted on postnatal survival or the general condition of the F1 offspring. 750 ppm (29 mg/kg/day) was considered an NOAEL for both maternal and offspring effects.

Study 3: In-vitro mode of action study in the rat, rabbit, and human (DAR B6.6.12.3).

Sulfoxaflor is a compound with insecticidal activity that acts as an agonist of insect nicotinic acetylcholine receptors (nAChRs). The aim of the work described in this report was to examine the influence of sulfoxaflor on mammalian muscle nAChRs. Competition radioligand binding was used to examine the ability of sulfoxaflor to bind to nAChRs from three mammalian species (human, rabbit and rat). In addition, two-electrode voltage-clamp recording was used (with human and rat nAChRs) to examine whether binding of sulfoxaflor resulted in functional activation of muscle nAChRs. Radioligand binding experiments demonstrated that sulfoxaflor binds to human, rabbit and rat foetal muscle nAChRs. Electrophysiological studies revealed that sulfoxaflor is a partial agonist of the rat foetal muscle nAChR. In contrast, sulfoxaflor has no detectable agonist activity on the human foetal muscle nAChR or on the adult muscle nAChR (from either human or rat). In contrast to the clear agonist activity of sulfoxaflor on the rat foetal muscle nAChR, no agonist activity was observed with X11719474, a soil metabolite of sulfoxaflor. This non-guideline study *in-vitro* mode of action study was considered acceptable.

Report:	XDE-208: Characterization of the agonist effects of XDE-208 on mammalian muscle nicotinic acetylcholine receptors.
Author:	Millar, N.
Date of Report:	7th June, 2010
Report Identity:	Study ID: UCL nAChR
Testing Facility:	Research Department of Neuroscience, Physiology & Pharmacology, University College London (London UK)
GLP	Signed and dated GLP (non-compliance) and (No) Data Confidentiality statements were provided. A Quality Assurance statement was not provided.
Test Substance:	XDE-208 (95.6% (wt/wt); as two diastereomers in 48.4/47.4% ratio. X11719474, a soil metabolite of XDE-208.
Batch:	E2162-34 XS9-37307-78
Guidelines:	Non-guideline
Deviations:	Not applicable
Acceptable:	Yes

Materials and Methods

Sulfoxaflor is a compound with insecticidal activity that acts as an agonist of insect nicotinic acetylcholine receptors (nAChRs). The aim of the work described in this report was to examine the influence of sulfoxaflor on mammalian muscle nAChRs. Competition radioligand binding was used to examine the ability of Sulfoxaflor to bind to nAChRs from three mammalian species (human, rabbit and rat). In addition, two-electrode voltage-clamp recording was used (with human and rat nAChRs) to examine whether binding of sulfoxaflor resulted in functional activation of muscle nAChRs.

Study design/Procedures

1. Animal husbandry and euthanasia

Time-mated sexually mature female rats [Crl:CD(SD) strain, Charles River Laboratories Inc.,

Portage, Michigan] and rabbits (New Zealand White strain, Covance Research Products Inc., Kalamazoo, Michigan) were supplied to The Dow Chemical Company (Toxicology and Environmental Research and Consulting, Midland, MI) on gestation day (GD) 15 or 19, respectively. The rats and rabbits were five to six months of age and weighed 280-320g or 2907-3165g, respectively, at the time of mating. Each animal was evaluated by a laboratory veterinarian to determine the general health status and acceptability for study purposes upon arrival at the laboratory (fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International - AAALAC International). The animals were housed one per cage in stainless steel cages with a pressure activated lixit valve-type watering system in rooms designed to maintain adequate conditions (temperature, humidity, and photocycle), until the tissue collection procedure. The animals were not treated with any test material and were fed standard laboratory diets (Rats-LabDiet Certified Rodent Diet #5002; Rabbits-LabDiet Certified Rabbit Diet #5325, PMI Nutrition International, St. Louis, Missouri). Non-woven gauze on the cage floor (rats) or a variety of stainless steel objects on the cage front (rabbits) was provided as environmental enrichment. On GD 21 (rats) or GD 28 (rabbits) the pregnant animals were euthanized via either carbon dioxide inhalation (rats) or intravenous injection of Beuthanasia-D (Schering Corporation, Kenilworth, New Jersey) (rabbits). All viable foetuses were euthanized by sublingual oral administration of sodium pentobarbital solution. Forelimb muscle tissue (bilateral) was dissected on ice, pooled on a half-litter basis, weighed and flash frozen in liquid nitrogen. All samples were stored in a freezer at -80ºC until shipment to University College London.

2. Preparation of tissue homogenates for radioligand binding

Tissue samples of foetal forelimb muscle isolated from rat and rabbit foetuses supplied by The Dow Chemical Company (Toxicology and Environmental Research and Consulting) were stored frozen until use (tissue samples were shipped on dry ice and subsequently stored in a freezer at -80ºC). Tissue was homogenized in ice-cold homogenization buffer (10 mM Tris-HCl buffer, pH 7.4, 1 mM EDTA, with protease inhibitors). The homogenate was centrifuged at low speed to remove debris and the supernatant collected. The supernatant was then centrifuged at high speed the pellet (corresponding to the cell membrane fraction containing the neuromuscular junction nAChRs) retained. Pellets were re-suspended in ice-cold homogenization buffer, aliquoted and then frozen rapidly by emersion of the tubes in an ethanol bath containing dry ice. Samples were stored at -80°C until required for radioligand binding experiments.

3. Mammalian cell culture, transfection and cell harvesting

Human embryonic kidney (HEK) cells were cultured in Dulbecco's modified Eagle’s medium (DMEM) containing 10% foetal calf serum, penicillin (100 U/ml) and streptomycin (100 mg/ml). Cells were maintained in a humidified incubator containing 5% CO2 at 37ºC. Cloned cDNAs encoding human nAChR subunits for the ‘foetal’ (α1)2β1δγ) and ‘adult’ (α1)2β1δε) muscle receptors in plasmid expression vector pcDNA3 were introduced into cultured HEK cells using Effectene reagent (Qiagen, Crawley, UK) according to the manufacturer’s instructions. After overnight incubation in Effectene, cells were incubated at 37ºC for 24 h before being harvested for radioligand binding. Cell monolayers were harvested by washing once in phosphate buffered saline (PBS) and then scraped into ice-cold PBS and pelleted by centrifugation. The culture supernatant was discarded and the pellet re-suspended in ice-cold 10 mM potassium phosphate buffer (pH 7.4) containing protease inhibitors. The suspension was taken up five times through a 23-guage needle to disrupt cells.

4. Radioligand binding and competition binding

Radioligand binding to muscle tissue homogenates or transiently transfected HEK cells was performed using [3H]-epibatidine (56 Ci/mmol; PerkinElmer Life and Analytical Sciences) or [3H]-sulfoxaflor (86 Ci/mmol; Dow AgroSciences). For competition binding experiments, a range of concentrations (0.3 µM to 30 mM) of unlabelled sulfoxaflor (lot number E2162-34 (95.6%); Dow AgroSciences) were used to displace a fixed concentration (30 nM) of [3H]-epibatidine. Triplicate samples were measured at each concentration and experiments were repeated at least three times using independently prepared tissue or cell preparations. Cell membranes and tissue homogenates (typically 80-150 mg protein) were incubated with radioligand for 150 min at 4ºC in a total volume of 300 µl in the presence of protease inhibitors, as described previously. Radioligand binding was assayed by rapid filtration onto Whatman GF/B filters (pre-soaked in 0.5% polyethylenimine), followed by rapid washing with cold 10 mM phosphate buffer using a Brandel cell harvester. Bound radioligand was quantified by scintillation counting using a Beckman LS6500 scintillation counter. Amounts of total cellular protein per sample in binding assays were determined using a Bio-Rad DC protein assay with BSA protein standards. Levels of specific radioligand binding were determined as mol/mg protein and curves for equilibrium binding fitted with the Hill equation using GraphPad Prism v. 4 (GraphPad Software, San Diego, CA, USA).

5. Microinjection of Xenopus oocytes with cDNA or cRNA

Mature oocytes were isolated from adult female *Xenopus laevis* and their follicular cell layer removed by treatment with collagenase type I (6 mg/ml) in calcium-free Barth's solution for 4 h at room temperature, followed by several washes in calcium-free Barth's solution. If necessary, collagenase treatment was followed by manual defolliculation. Oocytes were then injected with either cDNA (to express human nAChRs) or cRNA (to express rat nAChR subunits) using a Drummond variable volume microinjector. After injection, oocytes were incubated at 18ºC in Barth's solution for one to five days. The choice of cDNA or cRNA was dictated by the type of plasmid expression vector into which the human and rat nAChR subunits were cloned. Human nAChRs were cloned into plasmid pcDNA3 downstream from a cytomegalovirus (CMV) promoter that permits mRNA transcription when injected into the Xenopus oocyte nucleus. The rat nAChR subunits were cloned into plasmid pSPOoD which can be used for *in vitro* synthesis of mRNA (cRNA). The cRNA is injected into the oocyte cell cytoplasm, where it can be translated into protein.

6. In vitro synthesis of cRNA

Plasmid expression vectors constructs encoding nAChR subunits were linearized by restriction enzyme digestion. *In vitro* transcription, to generate cRNA, was then performed using the mMESSAGE mMACHINE SP6 transcription kit (Ambion, Huntington, UK). Reactions were carried out according to the manufacturer's protocol. Transcripts were recovered by precipitation with propan-2-ol, dissolved in nuclease-free water at a final concentration of 0.5 mg/ml and stored at -80ºC prior to use.

7. Expression of nAChRs in Xenopus oocytes

Two-electrode voltage-clamp recording from *Xenopus* oocytes was performed 1-5 days after micro-injection with cDNA or cRNA. Oocytes were placed in a recording chamber and continuously perfused with saline solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl2, 10 mM

Hepes, pH 7.3 with NaOH, 235 mOsm). Known agonists [X11719474 (lot number XS9-37307-78 (99.6%); Dow AgroSciences), Imidacloprid] or sulfoxaflor were applied using a computer-controlled perfusion system (BPS-8; ALA Scientific Inc., Westbury, NY). Two-electrode voltage clamp recording was performed using two microelectrodes filled with 3 M KCl and oocytes voltage clamped at –60 mV using an Axon Geneclamp 500B amplifier (Molecular Devices, Winnersh, UK). Membrane currents were digitized and stored on computer disk using pClamp software (Molecular Devices, Winnersh, UK).

Results and discussion

Radioligand binding:

Initial radioligand binding studies were performed with [^{3}H]-sulfoxaflor, the aim being to examine whether sulfoxaflor is able to bind to mammalian muscle nAChRs. Binding studies were performed with tissue homogenates prepared from rabbit and rat foetal forelimb muscle. Due to difficulty and concerns in obtaining and using human foetal muscle tissue, binding experiments with human nAChRs were performed with human recombinant nAChRs expressed in cultured human embryonic kidney (HEK) cells. Concentrations of [^{3}H]-sulfoxaflor up to 50 nM were examined but, due to high levels of non-specific binding, it was difficult to demonstrate conclusively whether [^{3}H]-sulfoxaflor bound specifically to these preparations. Technical difficulties were encountered in performing binding experiments with [^{3}H]-sulfoxaflor that were associated with high levels of non-specific binding, a problem that is often encountered with radioligands. Such problems are due to interaction of the radioligand with sites other than its receptor, for example with components of the cell (such as lipid membranes) or with the glass fiber filter that is used to assay the bound radioligand. Because of these technical difficulties, competition binding was employed to examine whether unlabelled sulfoxaflor was able to displace binding of the high-affinity nAChR radioligand [^{3}H]-epibatidine.

A series of experiments was performed to examine the ability of sulfoxaflor to displace [3H]-epibatidine (30 nM) from tissue homogenates prepared from rabbit foetal forelimb muscle, rat foetal forelimb muscle and human foetal recombinant $(\alpha1)2\beta1\gamma\delta$ nAChRs expressed in HEK cells. The ability of sulfoxaflor to displace binding of [3H]-epibatidine in rabbit foetal muscle tissue (A), rat foetal muscle tissue (B) and in HEK-293 cells expressing recombinant human foetal nAChRs (C) is shown in (Figure B6.6.12.3-1). The data are means + SEM of 3-4 independent experiments, each performed with triplicate samples. Levels of radioligand binding are normalized to the level of specific binding observed in the absence of sulfoxaflor. Concentrations are plotted as log molar concentrations

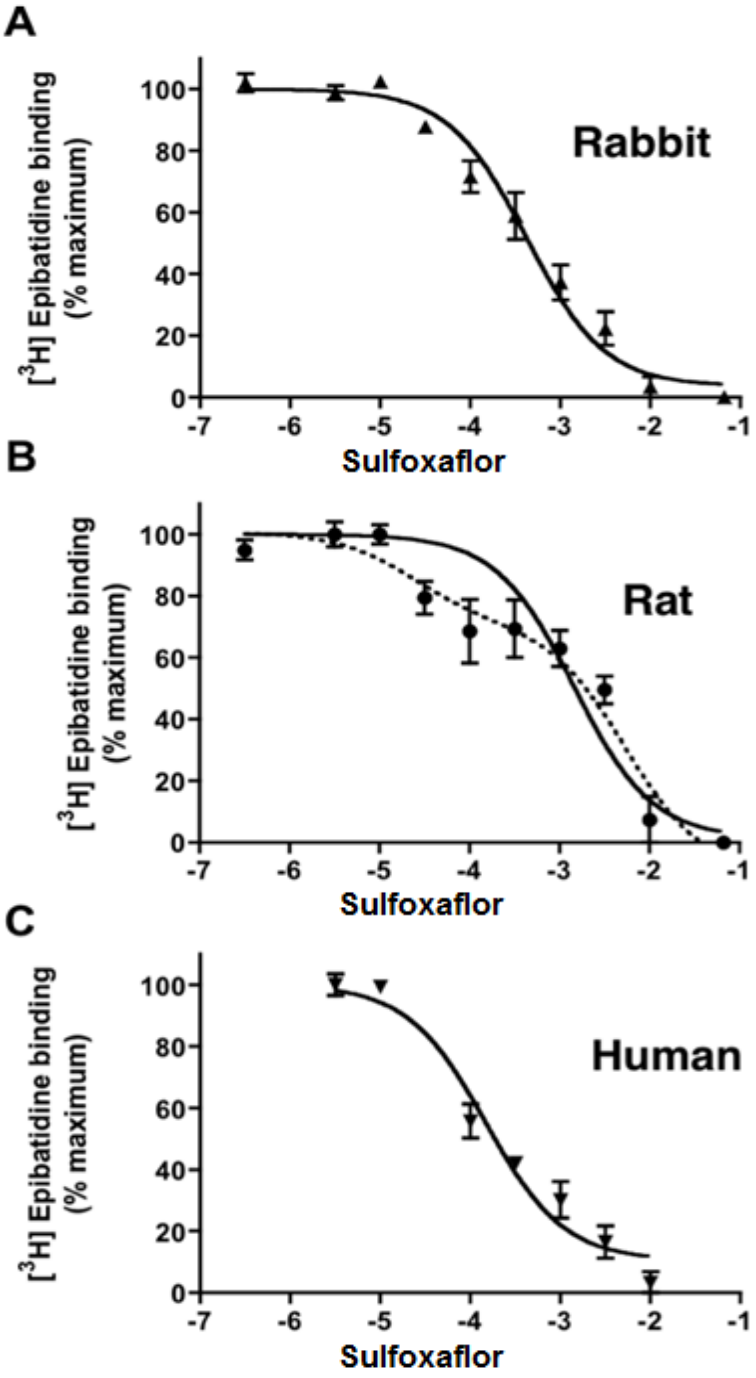


Figure 4.11.3.1.Study 3.1 (DAR Figure B.6.6.12.3-1): Competition Radioligand Binding with Sulfoxaflor

At higher concentrations of sulfoxaflor, almost complete displacement of bound [3H]-epibatidine was observed. Dose-dependent displacement of [^{3}H]-epibatidine binding was observed with increasing concentrations of sulfoxaflor for all three preparations examined (human, rabbit and rat).

Concentrations of sulfoxaflor causing half-maximal displacement (IC_{50} concentrations) of 30 nM [^{3}H]-epibatidine were determined. By fitting the data to a single binding site model (solid lines in Figure 1), estimates of IC_{50} for sulfoxaflor are 0.2 mM for human, 0.4 mM for rabbit and 2.3 mM for rat (Table B.6.6.12.3-1). Although data obtained with human nAChRs and with rabbit muscle are well fitted by the single-site model (and are also very similar to one another), this is not the case for rat foetal muscle. The binding data from rat muscle were, therefore, fitted with a two-site model (dotted line in Figure 1B) that revealed two distinct binding sites of different affinities (0.01 mM and 8.9 mM).

Table 4.11.3.1.Study 3.1 (DAR Table B.6.6.12.3-1): Sulfoxaflor displacement binding data

Species	IC_{50} (mM) ± SEM one-site model	IC_{50} (mM) ± SEM two-site model
Human	0.2 ± 0.03	
Rabbit	0.4 ± 0.1	
Rat	2.3 ± 0.5	0.01 ± 0.01 and 8.9 ± 3.1

The competition radioligand binding data obtained in this study provide clear evidence that sulfoxaflor binds to foetal muscle nAChRs. Complete displacement of specific [^{3}H]-epibatidine binding was observed with sulfoxaflor for all three preparations (human, rabbit and rat). However, it should be noted that, whereas the inhibition dissociation constant (K_i) for a ligand is a measure of the affinity of the ligand for its receptor, IC_{50} values (determined by equilibrium competition radioligand binding) are influenced by the concentration (A) of the radioligand ([$_3$H]-epibatidine) and also by the affinity of the radioligand for the receptor (its K_d). The relationship between IC_{50} and Ki can be expressed in the form of the 'Cheng Prusoff' equation:

$$K_i = \frac{IC_{50}}{1 + (A/K_d)}$$

As has been described elsewhere, epibatidine binds with very high (sub-nM) affinity to mammalian foetal muscle nAChRs. Thus, the IC_{50} values determined by competition with 30 nM [^{3}H]-epibatidine are likely to underestimate the affinity with which sulfoxaflor binds to muscle nAChRs. Conversion of IC_{50} values into K_i values is, however, complicated by the fact that epibatidine has been reported to bind to the two binding sites on the muscle nAChR with different affinities. This complicates conversion of IC_{50} data into K_i values and, for this reason, caution should be used when comparing IC_{50} data from the three nAChR preparations. The main conclusion that can be made from these binding studies is that sulfoxaflor is capable of causing complete displacement of [^{3}H]-epibatidine from all three species examined, thereby illustrating that sulfoxaflor binds specifically to nAChRs from all three species.

Functional Characterisation:

The radioligand binding experiments described above demonstrate specific binding of sulfoxaflor to muscle nAChRs but do not indicate whether the binding of sulfoxaflor results in

functional activation of the receptor (i.e., it does not indicate whether sulfoxaflor acts as an agonist on mammalian muscle nAChRs). To examine this question, human and rat foetal and adult muscle nAChRs were expressed as recombinant receptors by microinjection of cDNA/cRNA in Xenopus oocytes. This approach was possible because all five muscle nAChR subunits from both human and rat had been cloned and characterized previously. In contrast, there have been no reports of the molecular cloning of nAChR subunits from rabbit. For this reason, rabbit muscle nAChRs were not examined by this approach. Although, molecular cloning of cDNAs encoding the five rabbit muscle nAChR subunits is possible, this would require a considerable amount of additional work (perhaps 6-12 months for a skilled postdoctoral scientist), particularly since the nucleotide sequence of the nAChR subunit genes from rabbit are not known.

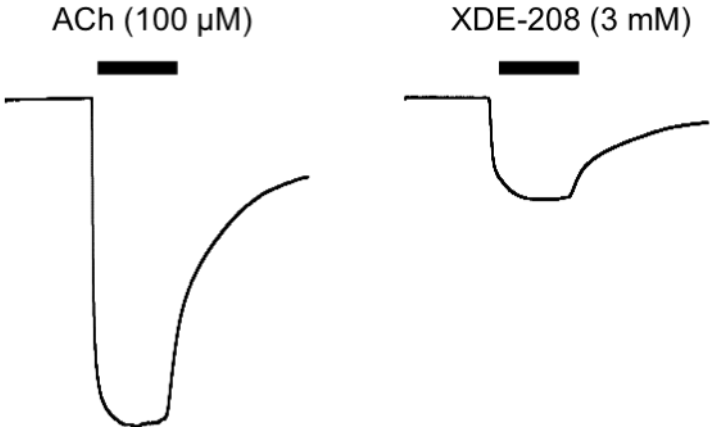
It should be noted that, although the nAChRs expressed in Xenopus oocytes are derived from cloned nAChR subunit cDNAs or cRNAs (rather than from mRNA in muscle cells), there is no reason to think that the electrophysiological data obtained from such receptors would be significantly different from data obtained from native nAChRs expressed in muscle tissue. In both cases (recombinant receptors in oocytes or native receptors in muscle), functional, fully assembled pentameric nAChRs are expressed on the cell surface. In the same way, recombinant muscle nAChRs expressed in cultured cell lines generate fully functional receptors with properties that mimic that of native receptors.

The ability of sulfoxaflor to act as an agonist of foetal and adult muscle nAChRs was examined by expression of rat and human nAChRs in *Xenopus* oocytes. This was achieved by microinjection of cDNA or cRNA encoding the appropriate rat or human muscle nAChR subunits. To generate the foetal form of the muscle nAChRs, α1, β1, γ and δ cDNAs or cRNAs were injected, whereas α1, β1, δ and ε were injected to generate the adult form. In each case, functional responses (membrane currents) were detected in response to application of the endogenous agonist acetylcholine (ACh). An example of a current recorded in response to the application of ACh to rat (α1)2β1γδ nAChRs is shown in Figure B.6.6.12.3-2. As can be seen from the representative traces presented, muscle nAChRs remain open during prolonged agonist application (the receptor undergoes no discernable desensitisation). This is a feature that is not shared by all nAChRs. For example, nAChRs such as the "neuronal" α7 nAChR, undergo very rapid desensitization after initial agonist activation.

Figure B.6.6.12.3-2 represents whole-cell current responses in a *Xenopus* oocyte cell expressing foetal rat (α1)2β1γδ nAChRs. Inward currents are shown from the same oocyte in response to application of acetylcholine (100 μM) and sulfoxaflor (3 mM). The length of agonist application (5 secs.) is indicated by the horizontal bar. Agonist activation is associated with downward deflection in the trace. Note recovery of the response after acetylcholine or sulfoxaflor is washed off.

Figure 4.11.3.1.Study 3.2 (DAR Figure B.6.6.12.3-2): Representative whole-cell current responses of the foetal rat (α1)2β1γδ nAChRs

Representative data showing ACh dose-response curves at human and rat foetal and adult muscle



nAChRs are shown in Figure B.6.6.12.3-3. The ability of sulfoxaflor to generate agonist-evoked currents was also examined in *Xenopus* oocytes expressing rat and human nAChRs. Clear agonist-evoked responses were observed with sulfoxaflor at the rat foetal nAChR. Sulfoxaflor acted as a relatively low potency (EC_{50} > 0.6 mM) partial agonist (the maximum response observed with sulfoxaflor at 3 mM was 39 ± 2.4% of that detected with a maximal concentration of ACh) (Table B.6.6.12.3-1.). Due to limits of solubility, the highest concentration of sulfoxaflor tested was 3 mM. In contrast to its effect at the rat foetal muscle nAChR, no agonist activity of sulfoxaflor was observed at the human foetal muscle nAChR (Table B.6.6.12.3-1, Figure B.6.6.12.3-3) up to the maximum feasible concentration (3 mM) despite normal ACh agonist responses. Similarly, no agonist effect of sulfoxaflor was observed with the adult rat or human muscle nAChR, despite normal ACh agonist responses.

Figure 4.11.3.1.Study 3.3 (DAR Figure B.6.6.12.3-3): Agonist activation of nAChRs

expressed in *Xenopus* oocytes.

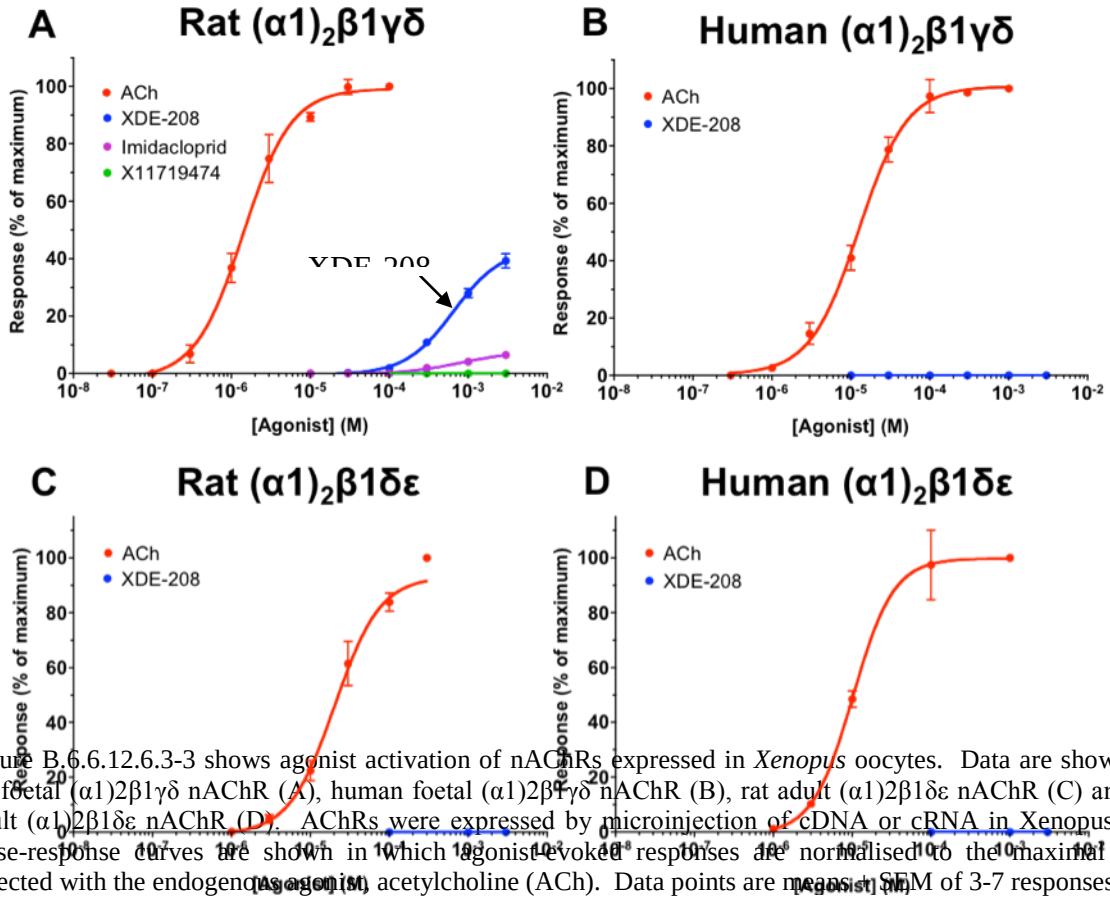


Figure B.6.6.12.6.3-3 shows agonist activation of nAChRs expressed in *Xenopus* oocytes. Data are shown for the rat foetal (α1)2β1γδ nAChR (A), human foetal (α1)2β1γδ nAChR (B), rat adult (α1)2β1δε nAChR (C) and human adult (α1)2β1δε nAChR (D). AChRs were expressed by microinjection of cDNA or cRNA in Xenopus oocytes. Dose-response curves are shown in which agonist-evoked responses are normalised to the maximal response detected with the endogenous agonist, acetylcholine (ACh). Data points are means + SEM of 3-7 responses.

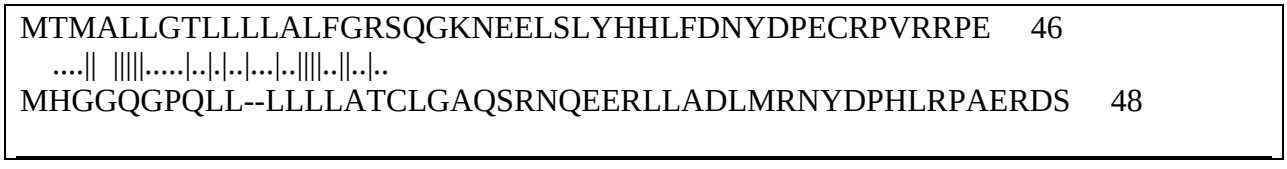
Comparison of the amino acid sequence of the rat and human γ subunit revealed that, although the two subunits are similar (approximately 90% identical), they contain 53 amino acid differences (see Figure B.6.6.12.6.3-4). Given the evidence (described above) that one or two amino acid differences can confer species-selective agonist activity upon nicotinic ligands, it seems entirely plausible that the differences in agonist activity of XDE-208 can be explained by differences in the amino acid sequence of the rat and human nAChR γ subunits. The γ and ε subunits show even greater sequence differences that the human and rat γ subunit (even from the same species). Figure 5 shows an alignment of the γ and ε subunits from rat. These subunits share only about 50% identity in amino acid sequence.

```
MHGGQGPLLLLLLLAVCLGAQGRNQEERLLADLMQNYDPNLRPAERDSDV   50
|||||||.|||||||.|||||.||||||||||||.||||.||||||||||
MHGGQGPQLLLLLLLATCLGAQSRNQEERLLADLMRNYDPHLRPAERDSDV   50

VNVSLKLTLTNLISLNEREEALTTNVWIEMQWCDYRLRWDPRDYEGLWVL   100
||||||||||||||||||||||||||||||||||||||||||||||.|||||.|
VNVSLKLTLTNLISLNEREEALTTNVWIEMQWCDYRLRWDPKDYEGLWIL   100

RVPSTMVWRPDIVLENNVDGVFEVALYCNVLVSPDGCIYWLPPAIFRSAC   150
```

Figure 4.11.3.1.Study 3.4 (DAR Figure B.6.6.12.6.3-4): Sequence alignment of the rat and human nAChR γ subunits. An alignment is shown of the amino acid sequences of the human (top line) and rat (bottom line) nAChR γ subunits. Amino acids that are conserved between the two subunits are indicated by a vertical line (|). Amino acid differences are indicated by a dot (.). Gaps that have been inserted in one or other sequence in order to obtain an optimal alignment are indicated by a dash (-). Amino acids are indicated by standard one-letter abbreviations and are numbered from the first methionine (M) of the N-terminal signal sequence.



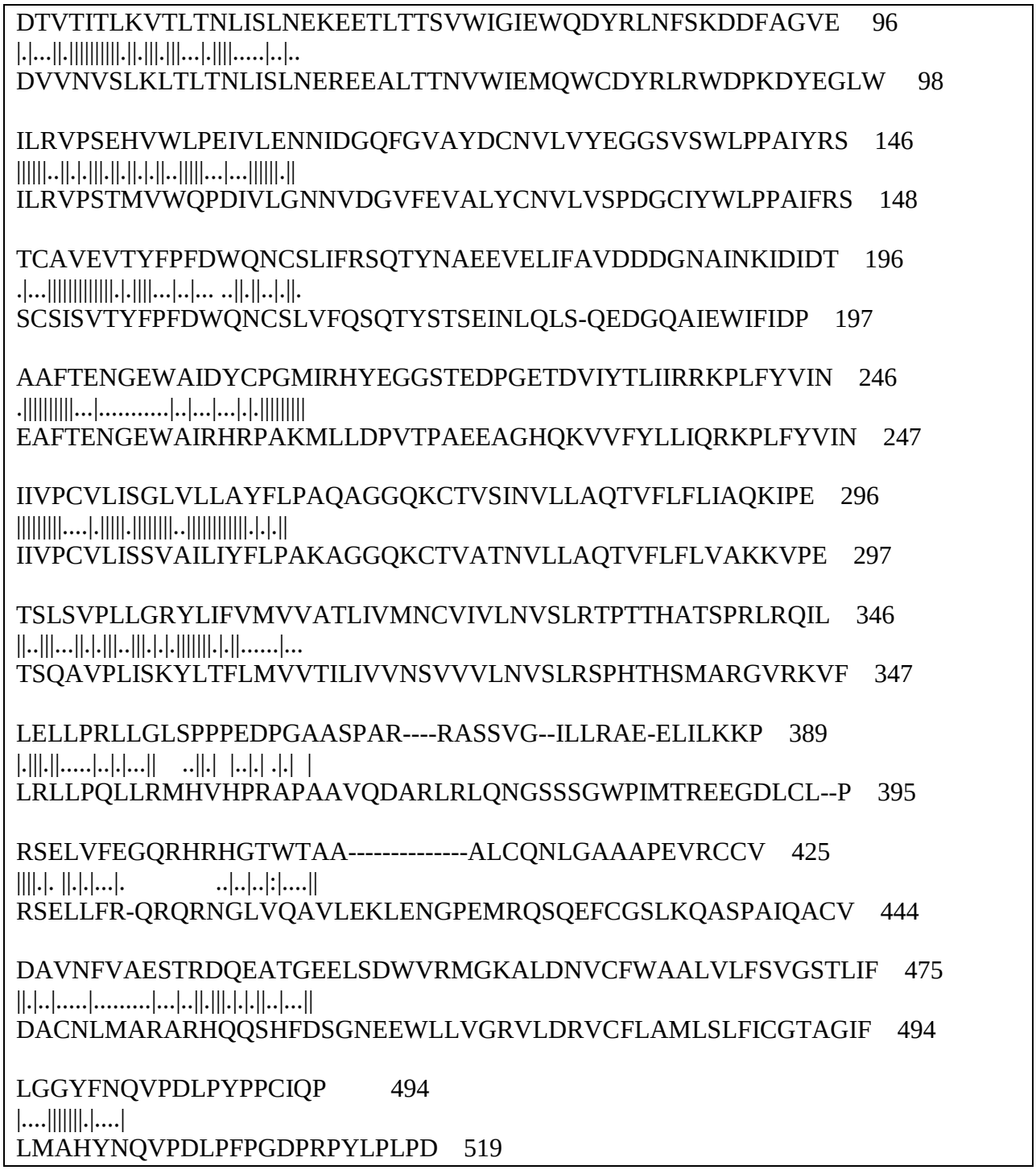


Figure 4.11.3.1.Study 3.5 (DAR Figure B.6.6.12.6.3-5.): Sequence alignment of the rat nAChR γ and ε subunits**:** An alignment is shown of the amino acid sequences of the rat ε (top line) and rat γ (bottom line) nAChR subunits. Amino acids that are conserved between the two subunits are indicated by a vertical line (|). Amino acid differences are indicated by a dot (.). Gaps that have been inserted in one or other sequence in order to obtain an optimal alignment are indicated by a dash (-). Amino acids are indicated by standard one-letter abbreviations and are numbered from the first methionine (M) of the N-terminal signal sequence.

Conclusions:
The work described in the present study demonstrates that sulfoxaflor is an agonist of the rat foetal muscle nAChR (which contains the rat γ subunit). In contrast, sulfoxaflor has no agonist activity on the equivalent human nAChR (containing the human γ subunit) or on the rat or human adult muscle nAChR (containing the rat or human ε subunit). From these findings, it seems reasonable to conclude that the selective agonist activity of sulfoxaflor is due to differences in the amino acid sequence of the rat γ subunits compared with that of the human γ subunit (and also with the rat and human ε subunit).

Study 4: Critical window Phase 1
Exposure to 1000 ppm sulfoxaflor throughout gestation (gestation days (GD) 6-21) has been previously shown to cause foetal limb contractures (forelimb flexure and hindlimb rotation) and reduced neonatal survival. It was hypothesised that these effects might result from agonism of sulfoxaflor at the foetal muscle nicotinic acetylcholine receptor (nAChR) based on information available at the time, which indicated 1) this is consistent with the molecule's insecticidal mode-of-action, 2) a soil metabolite of sulfoxaflor (X11719474), which does not bind to the insect nAChR, did not induce limb contractures or reduced neonatal survival even at very high dose levels, and 3) this muscle receptor subtype is highly expressed during late gestation in the distal limbs muscles and diaphragm, with impairment of diaphragmatic maintenance of respiration at birth implicated in neonatal death from sulfoxaflor exposure.
This was the first of two studies conducted to determine the critical window of susceptibility, and to test the hypothesis that late gestational exposure to sulfoxaflor induces foetal abnormalities and reduced neonatal survival *via* its pharmacological action on the foetal muscle nicotinic acetylcholine receptor (nAChR). This receptor develops functional expression between GD 16 and 17 in the rat, resulting in synchronised foetal limb movements and diaphragmatic responsiveness important for the transition to extrauterine respiration.
In this study, groups of 12 female Crl:CD(SD) rats were administered control diet (Group 1), or diets containing 1000 ppm sulfoxaflor fed from GD 6-16 (Group 2) to cover all of embryogenesis up to, but not including, the start of early foetal movements, or 1000 ppm sulfoxaflor fed from GD 16-birth (Group 3) to cover development of the muscle nAChR and its role in development of synchronised foetal limb movements up to onset of parturition. In the offspring, effects on litter size, survival, body weight and the presence of gross external morphological alterations, with particular focus on limb abnormalities (e.g., forelimb flexure and hindlimb rotation), were carefully assessed. In addition, a subset of animals was examined for the presence of convoluted ureters and bent clavicles as these effects had also been seen in the sulfoxaflor rat developmental toxicity study at 1000 ppm.
Offspring from animals given 1000 ppm sulfoxaflor from GD 6-16 (Group 2) were completely normal and did not display previously described foetal abnormalities or reduced neonatal survival. In contrast, offspring given 1000 ppm sulfoxaflor from GD 16-birth (Group 3) had the same gross effects of limb contractures and reduced neonatal survival seen in the previous studies that had treatment with 1000 ppm sulfoxaflor throughout

gestation. This demonstrates that the critical window of susceptibility for both of these effects falls within GD 16-birth.

In addition, daily examination of Group 3 offspring born with limb abnormalities indicated that these were fully reversible shortly after withdrawal of maternal dietary exposure to sulfoxaflor. In some cases, full reversal of the limb abnormalities was evident the day after birth and occurred for all affected animals that survived to postnatal day (PND) 4; reversal also occurred in some animals that subsequently died before PND 4. Likewise, the visceral and skeletal findings of abnormal ureter and bent clavicle, the latter of which had a high incidence (30.1% of fetuses), in the definitive developmental toxicity study were not present in this study at necropsy on PND 4 despite similar blood concentrations and limb abnormality indices between these two studies.

In summary, this study demonstrated that the critical period of developmental susceptibility to sulfoxaflor-induced foetal abnormalities and reduced neonatal survival is between GD 16-birth, and that all of the foetal abnormalities are rapidly reversible after birth. These results support the hypothesis that late gestational exposure to sulfoxaflor induces foetal abnormalities and neonatal death *via* its pharmacological action on the foetal muscle nicotinic acetylcholine receptor (nAChR), which develops functional expression during this stage of gestation. This non-guideline study is acceptable.

Report:	XDE 208: Investigation of the critical window of exposure for fetal abnormalities and neonatal survival effects in Crl:CD(SD) rats.
Author:	Rasoulpour, R. and C. Zablotny
Date of Report:	**2**5th June, 2010
Report Identity:	Study ID: 091022
Testing Facility:	Toxicology & Environmental Research and Consulting, The Dow Chemical Company (Michigan).
GLP	Signed and dated GLP (non-compliance), Quality Assurance, and (No) Data Confidentiality statements were provided. .
Test Substance:	XDE-208 (95.6% (wt/wt); as two diastereomers in 48.4/47.4% ratio.
Batch:	E2162-34
Guidelines:	Non-guideline
Deviations:	Not applicable
Acceptable:	Yes

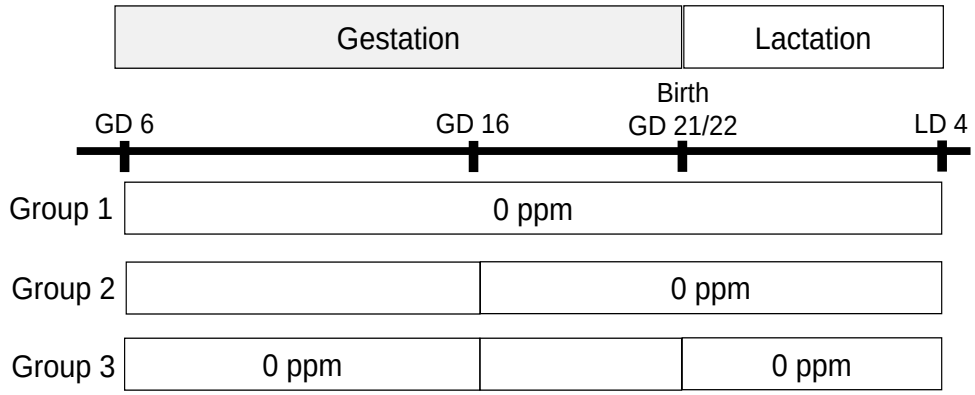
Materials and Methods

In a non-guideline reproductive/developmental toxicity study sulfoxaflor was administered to pregnant female Crl:CD(SD) rats (12/group) at concentrations of 0 (Group 1, control) or 1000 ppm (Groups 2 and 3) in the diet (corresponding to doses of 0, 38.6, and 76.5 mg/kg/day for Groups 1, 2, and 3, respectively). Group 2 was fed the test substance from gestation day (GD) 6-16 to cover all of embryogenesis up to, but not including, the start of early foetal movements; Group 3 was fed the test substance from GD 16-birth to cover development of the muscle nAChR and its role in development of synchronized foetal limb movements up to onset of parturition.

Table 4.11.3.1.Study 4.1 (DAR Table B.6.6.12.4-1.): Treatment Groups

Group	Sulfoxaflor Treatment Period	No. of Rats
1	N/A (Control)	12
2	GD 6-16	12
3	GD 16-parturition	12

Figure 1



Group 1, the control group, received control feed (0 ppm) from GD 6 until termination on lactation day (LD) 4. Group 2 was administered feed containing 1000 ppm sulfoxaflor from GD 6 until the morning of GD 16, and was switched to control feed (0 ppm) until termination on LD 4. Group 3 was administered control feed (0 ppm) from GD 6 until the morning of GD 16; feed containing 1000 ppm sulfoxaflor was offered from GD 16 until parturition was first observed during the daily clinical observations at approximately 7AM and 3PM (GD 21 or 22), and were switched back to control feed (0 ppm) until termination on LD 4.

The key study parameters and study schedule are outlined in Table B.6.6.12.4-2, and discussed in additional detail in the following subsections.

Table 4.11.3.1.Study 4.2 (DAR Table B.6.6.12.4-2.): Summary of Key Study Parameters and Study Schedule

Study Events and Parameters	No. Animals[a]	Timing
Cage-side examinations	All[b]	At least twice daily
Clinical observations	All	Conducted on GD 6, 9, 12, 15, 17 and 20; LD 0-4
Maternal body weights	All	Recorded on GD 0 (by the supplier), GD 3, daily from 6-9, 12, 14, daily from 16-21; and LD 1 and 4 for females delivering a litter
Feed consumption	All	Recorded on GD 3-6, daily from GD 6-9, 12-14, 14-16, daily from GD 16-21; and LD 1 and 4 for females delivering a litter
Maternal blood collection	4/group	On GD 16 for Groups 1 and 2, and on GD 21 for Groups 1 and 3
Litter observations	All	Daily from PND 0-4
No. of live & dead	All	PND 0-4

Study Events and Parameters	No. Animals[a]	Timing
pups		
Pup body weights	All	PND 1 and 4
Termination of pups	All	PND 4
Gross necropsy – adult females	All	LD 4 or at least 24 days after evidence of mating for females not delivering a litter

[a]Adults unless specified otherwise; [b]includes dams and their litters; GD = gestation day, LD = lactation day, PND = postnatal day

Dose selection rationale: The dose level of 1000 ppm was based on the results of previous reproductive toxicity screening and developmental toxicity studies. This dose level was shown to decrease neonatal growth and survival and to produce foetal abnormalities.

Results

Dietary analysis: Analyses of the 1000 ppm diet, plus control and premix, revealed concentrations ranging from 94.6% to 95.9% of the targeted concentration. Analysis of aliquots of the 1000 ppm diet indicated that the test material was homogeneously distributed, based on a relative standard deviation (RSD) of 3.2%.

Maternal Observations

Mortality/clinical signs: All maternal animals survived until termination.

Body weight: Animals given 1000 ppm sulfoxaflor from GD 6-16 (Group 2) had treatment-related decreases in body weight from GD 7-21, resulting in decreased mean body weight gain from GD 6-16 relative to controls (Group 1). Similarly, animals given 1000 ppm sulfoxaflor from GD 16-birth (Group 3) had treatment-related decreases in body weight and mean body weight gain during test material administration intervals. These body weight effects were accompanied by lower feed consumption and consistent with effects at 1000 ppm in the definitive developmental toxicity study (B.6.6.12.2)). Body weight effects in Groups 2 and 3 from gestational treatment resulted in lower mean lactation body weights than controls on LD 1 and 4 . Mean maternal body weight and body weight gains during gestation are summarised in Table B.6.6.12.4-3.

Table 4.11.3.1.Study 4.3 (DAR Table B.6.6.12.4-3.): Maternal body weight change

Maternal Gestation Body Weight Gains[a]			
	Mean Body Weight Gain (grams±SD)		
Group:	**1 (n=9)**	**2 (n=10)**	**3 (n=12)**
Treatment period:	**Control**	**GD 6-16**	**GD 16-birth**
GD 6-9	14.1±6.6	**-2.0*** ±5.8	12.4±8.7
GD 16-17	10.6±2.8	17.1*±5.3	**5.6**±5.8
GD 19-21	29.4±8.2	**23.5**±6.0	**17.2***±9.7

GD 0-21	181.4±±22.7	**143.9*±**14.8	**142.9*±**36.5
GD 6-16	75.1±16.9	**50.2*±**6.9	**67.6±**16.8
GD 16-21	70.6±10.1	**65.6±**10.8	**43.4*±**18.9
Maternal Lactation Body Weights[a]			
	Mean Body Weight (grams±SD)		
Group:	**1 (n=9)**	**2 (n=10)**	**3 (n=12/7[a])**
Treatment period:	**Control**	**GD 6-16**	**GD 16-birth**
LD 1	315.8±19.3	298.1±15.5	297.0±23.0
LD 4	333.7±19.7	309.3*±18.1	308.5±28.8

* Statistically different from control mean by Dunnett's test, alpha = 0.05.
Results in bold type indicate the effects judged to be treatment-related.
[a] n=12 for LD 1; n=7 for LD 4 due to loss of litter

Food consumption/test substance intake: There was a treatment-related decrease in feed consumption in Groups 2 and 3 that corresponded to their different treatment periods, with individual intervals reaching statistical difference from controls. In comparing feed consumed during treatment with 1000 ppm sulfoxaflor, mean feed consumption from GD 6-16 in Group 2 and GD 16-21 in Group 3 were 23.3% and 22.6% lower than controls (Group 1) during these intervals. Although there was no test material administration during the lactation phase of the study, lactation feed consumption in Groups 2 and 3 were lower than Group 1 and statistically identified on LD 1-4 in Group 3. Mean feed consumption during gestation and LD 1-4 is presented in Table B.6.6.12.4-4.

Table 4.11.3.1.Study 4.4 (DAR Table B.6.6.12.4-4.): Mean maternal food consumption during gestation and lactation

Gestation Feed Consumption			
	Mean grams/animal/day relative to controls (%)		
Group:	**1 (n=9)[c]**	**2 (n=10)[a]**	**3 (n=12)**
Treatment period:	**Control**	**GD 6-16**	**GD 16-birth**
GD 6-16	100	**76.7**	98.3
GD 16-21	100	94.3	**77.4**

Lactation Feed Consumption			
	Mean grams/animal/day (±SD)		
Group:	**1 (n=9)**	**2 (n=10)**	**3 (n=6)**
Treatment period:	**Control**	**GD 6-16**	**GD 16-birth**
LD 1-4	36.0±5.3	**31.1**±5.1	**25.5***±6.8

Results in bold type indicate the effect judged to be treatment-related.
* Statistically different from control mean by Dunnett's test, alpha = 0.05.
[a] For GD 20-21, n=8 for Group 1 and n=9 for Group 2 because of exclusion of negative or scratched feed data.

Time-weighted average doses for animals receiving 1000 ppm sulfoxaflor were 76.5±5.6 mg/kg/day in Group 2 and 38.6±9.7 mg/kg/day in Group 3. The lower dose received in Group 3 represents a combination of increased body weight and slight decreases in feed consumption that occur near the end of gestation.

Table 4.11.3.1.Study 4.5 Table B.6.6.12.4-5. Test Material Intake (mg/kg/day)

GROUP:	**1**	**2 (n=10)**	**3 (n=12)**
Treatment period:	**Control**	**GD 6-16**	**GD 16-birth**
GD 6-16	0	76.5±5.6[a] (65.7-86.1)	0
GD 16-21	0	0	38.6±9.7 (20.8-53.8)

Necropsy: There were no treatment-related gross pathologic observations of animals at any dose level.

Toxicokinetic data: There was no sulfoxaflor found in plasma obtained from dams of the control group. The measured plasma concentrations of Groups 2 and 3 ranged from 35.4-40.9 and 32.1-43.2 µg sulfoxaflor /gram plasma, respectively. The concentrations of sulfoxaflor measured in maternal blood on GD 16 and 21 are summarised in Table B.6.6.12.4-5.

Table 4.11.3.1.Study 4.6 (DAR Table B.6.6.12.4-6.): Toxicokinetic Data

	Measured sulfoxaflor Maternal Blood Concentration (µg/g plasma)		
Group:	**1 (n=4)**	**2 (n=4)**	**3 (n=4)**
Treatment period:	**Control**	**GD 6-16**	**GD 16-birth**
GD 16	<LLQ	38.9±2.6 (35.4-40.9)	N/A
GD 21	<LLQ	N/A	38.2±4.6 (32.1-43.2)

Offsring observations

Reproductive indices/pup survival/sex ratio: There were no treatment-related effects on gestation survival or sex ratios.

Administration of 1000 ppm sulfoxaflor during gestation days (GD) 6-21 had previously been shown to induce decreased pup survival between PND 0-4. In this study, treatment-related effects on pup survival were limited to Group 3 litters that were exposed to 1000 ppm sulfoxaflor from GD 16-birth. It is important to note that there was no effect on pup survival in Group 2 dams, which were administered 1000 ppm sulfoxaflor from GD 6-16. These data provide clear differentiation between periods of sensitivity and insensitivity to the effect, which coincides with the expression of the foetal neuromuscular junction nAChR in rats and the resultant foetal limb movement synchronisation and diaphragmatic responsivity between GD 16 and 17, thus supporting the hypothesis that late gestational exposure to sulfoxaflor induces foetal abnormalities and reduced neonatal survival *via* its pharmacological action on the foetal muscle nicotinic acetylcholine receptor (nAChR). The responsitivity of the diaphragm is particularly important for the transition to extrauterine respiration. Rhythmic coordinated contractions of the respiratory muscles, mainly driven by the diaphragm, are observable by GD 18. The effects on pup survival are presented in Table B.6.6.12.4-7.

Table 4.11.3.1.Study 4.7 (DAR Table B.6.6.12.4-7.): Effects on Pup Survival

Mean litter size/% of litter size at birth			
Group:	**1**	**2**	**3**
Treatment period:	**Control N = 9**	**GD 6-16 N = 10**	**GD 16-birth N = 12**
PND 1 Survival	13.6/100.0	12.0/100.0	**9.9/83.2***
PND 2 Survival	13.6 /100.0	12.0/100.0	**5.9/49.7***
PND 3 Survival	13.4 /99.20	12.0/100.0	**5.7/47.6***
PND 4 Survival	13.3/98.4	11.9/100.0	**5.6/46.9***

* Statistically different from control mean by Censored Wilcoxon Test, alpha = 0.05.

Results in bold type indicate the effects judged to be treatment-related.

The pup survival incidence observed with Group 3 in this study is consistent with the previous OECD 421-like reproduction/developmental toxicity screening study (B.6.6.1/1), where a dose of 30.3 mg/kg/day (500 ppm) resulted in 81.2% pup survival (18.8% pup death) and 62.0 mg/kg/day (1000 ppm) resulted in 7.3% pup survival (92.7% pup death). The dose achieved with Group 3 dams in this study of 38.6 mg/kg/day resulted in 46.9% pup survival (53.1% pup death). These data indicate that a longer duration of exposure in the OECD 421-like study (exposure from two-weeks pre-breeding through the end of lactation) did not result in a more robust effect than GD 16-birth exposure when normalized to mg/kg/day dose. The results of the previous and current studies are compared in Table B.6.6.12.4-8.

Table 4.11.3.1.Study 4.8 (DAR Table B.6.6.12.4-8.): Pup Survival Comparison

	OECD 421-Like Study			**Critical Window 1 (current study)**
Dose (ppm)	100	500	1000	Group 3 1000
Treatment period	Two weeks prior to breeding - PND 21			GD 16-birth
Test Material Intake (mg/kg/day)	GD 14-20 6.07	GD 14-20 30.3	GD 14-20 62.0	GD 16-birth 38.6
TK plasma conc (ug SULFOXAFLOR/g)	N/A[A]	N/A[B]	LD4 14.3 - 41.9	GD21 32.1 - 43.2
PND 4 pup survival [b] Litter size/% of PND 0	14.6/97.8%	11.2/**81.2%**	**1.6/7.3%**	**5.6/46.9%**
Pup death [b]	2.2%	**18.8%**	**92.7%**	**53.1%**

Results in bold type indicate the effects judged to be treatment-related.
A, B = Tk data not collected, but for comparison LD4 blood levels from two-generation reproduction study were 3.84-5.15 ug/g plasma at 100 ppm and 14.9-16.6 ug/g plasma at 400 ppm

Litter size and pup body weights: There were no treatment-related differences in the number of pups born alive or dead in Groups 2 or 3 when compared to the control (Group 1). Consistent with the previously described effects, Group 3 offspring postnatal survival was lower than Groups 1 and 2. Litter size data are presented in Table B.6.6.12.4-9.

Table 4.11.3.1.Study 4.9 (DAR Table B.6.6.12.4-9.): Litter Size

	Mean Litter Size

	1(n=9)	2 (n=10)	3 (n=12)
Treatment	**Control**	**GD 6-16**	**GD 16-**
Born live	13.6±2.1	12.0±0.9	11.9±3.7
Born dead	0.4±1.3	0.2±0.4	0.3±0.5
LD 1	13.6±2.1	12.0±0.9	**9.9**±4.7
LD 2	13.6±2.1	12.0±0.9	**5.9*** ±6,1
LD 3	13.4±2.0	12.0±0.9	**5.7*** ±6.0
LD 4	13.3±1.9	11.9±1.1	**5.6*** ±6.1

*Statistically different from control mean by Wilcoxon's Test, alpha = 0.05.
Bold type indicates the effects judged to be treatment related.

In addition to effects on pup survival and consistent with previous studies, there was a treatment-related 18.8-20.8% decrease in Group 3 pup body weight in both sexes, relative to controls (Table B.6.6.12.4-10). There were no treatment-related effects on the body weight of offspring in Group 2 when compared to the control group.

Table 4.11.3.1.Study 4.10 (DAR Table B.6.6.12.4-10): Mean Pup body weights

	Group		
Pup Age and Sex	1	2	3
Treatment period	Control	GD 6-16	GD 16-birth
PND 1 Female	6.8	7.0	**5.5***
PND 1 Male	7.2	7.3	**5.7***
PND 4 Female	10.1	10.3	**8.1***
PND 4 Male	10.6	10.5	**8.6***

* Statistically different from control mean by Wilcoxon's Test, alpha = 0.05.
Bold type indicates the effects judged to be treatment related.

Toxicokinetic data: There was no sulfoxaflor found in plasma obtained from dams of the control group. The measured plasma concentration of Groups 2 and 3 ranged from 35.4-40.9 and 32.1-43.2 µg sulfoxaflor, respectively.

Pathology

Pup alterations: Offspring from dams given 1000 ppm sulfoxaflor from GD 16-birth (Group 3) were observed with limb abnormalities of forelimb flexure and hindlimb rotation, as previously identified in the definitive developmental toxicity study in rats given 1000 ppm from GD 6-21. On the day of birth (PND 0) 35% of pups from 91.7% of litters were observed with forelimb flexure, while 13.3% of pups from 66.7% of litters were observed with hindlimb rotation. This incidence of limb abnormalities decreased through PND 1-3 due to a combination of pup death and reversibility of the limb abnormalities (see Reversibility section), as evidenced by a 0% incidence of forelimb flexure and hindlimb rotation by PND 4. Similar to the pup survival

effects, offspring from Group 2 (treated GD 6-16) were unaffected, further supporting the clear differentiation between periods of sensitivity and insensitivity to the effect, which coincides with the expression of the foetal neuromuscular junction nAChR in rats.

Table 4.11.3.1.Study 4.11 (DAR Table B.6.6.12.4-11): Limb alterations in Group 3

Postnatal Day	Forelimb Flexure No. affected/Total No. (percent)		Hindlimb Rotation No. affected/Total No. (percent)	
	Pups	Litter	Pups	Litter
PND 0	50/143 (35.0)	11/12 (91.7)*	19/143 (13.3)	8/12 (66.7)*
PND 1	38/119 (31.9)	9/12 (75.0)*	8/119 (6.7)	6/12 (50.0)*
PND 2	6/71 (8.5)	4/8 (50.0)*	2/71 (2.8)	2/8 (25.0)
PND 3	0/68 (0.0)	0/7) (0.0)	1/68 (1.5)	1/7 (14.3)
PND 4	0/67 (0/0)	0/7 (0/0)	0/49 (0.0)	0/7 (0/0)

* Statistically different from control mean by Wilcoxon's Test, alpha = 0.05.

On PND 4, one pup/sex/litter from Groups 1 and 2 and all available pups from Group 3 underwent visceral examination to determine the presence or absence of convoluted ureters and skeletal examination for bent clavicles, which were identified as treatment-related effects in the definitive developmental toxicity study at 1000 ppm sulfoxaflor. There were no observations of convoluted ureters or bent clavicles in the 18 and 20 pups evaluated in Groups 1 and 2, respectively. Despite the fact that offspring in Group 3 exhibited treatment-related limb contractures (forelimb flexure and hindlimb rotation), there were also no observations of convoluted ureters or bent clavicles in the 49 pups evaluated from this group.

Reversibility: As mentioned above, offspring from dams given 1000 ppm sulfoxaflor from GD 16-birth (Group 3) were observed on the day of birth (PND 0) with forelimb flexure (35% of pups from 91.7% of litters) and hindlimb rotation (13.3% of pups from 66.7% of litters); however, by PND 4 there were no incidences of either limb abnormality. In order to determine if this absence of findings on PND 4 was due to affected pups dying *vs.* reversals of these limb abnormalities, a table was created tracking the fate of each litter with respect to the number of pups alive, number of pups dead, and number of pups with limb abnormalities on each day. In analysing the data it became apparent that the limb abnormalities must have reversed in some pups. For example, dam 714 gave birth to 14 live pups of which six had limb abnormalities on PND 0. On the next day, there were still 14 live pups, but only two had limb abnormalities, which indicate that four pups recovered from these limb abnormalities between PND 0 and 1. In total, all 21 pups with limb abnormalities whose subsequent reversibility could be tracked with certainty indeed had reversed between PND 0 and 4 (Table B.6.6.12.4-11). This represents the minimum number of potential reversals, as some might have occurred but were not detected prior to death. These data indicate that the limb abnormalities were transient alterations, consistent with a pharmacologic mode-of-action of sulfoxaflor on the limb muscles. The limb abnormality reversal data are summarised in Table B.6.6.12.4-12

Table 4.11.3.1.Study 4.12 (DAR Table B.6.6.12.4-12): Limb Abnormality Reversals

	Number of Reversals (pups/day)		
Group:	**1**	**2**	**3**
Treatment period:	**Control**	**GD 6-16**	**GD 16-birth**
PND 0 to 1	N/A	N/A	11
PND 1 to 2	N/A	N/A	6
PND 2 to 3	N/A	N/A	3
PND 3 to 4	N/A	N/A	1
Total Reversals	N/A	N/A	**21**

N/A = not applicable

In addition to reversal of the limb abnormalities, there were no observations of convoluted/hydroureter ureters or bent clavicles at PND 4, despite the fact that these findings were observed on GD 21 foetuses from dams exposed to 1000 ppm sulfoxaflor in the developmental toxicity study (), and at a relatively high incidence (30.1% of foetuses) for bent clavicles. Foetal convoluted/hydroureter are variants that result from a temporary build-up of urine in the ureter and are known to be readily reversible after birth. Postnatal remodeling of skeletal abnormalities has been shown with other test compounds such as caffeine and ethylene glycol.

Comparison to Developmental Toxicity Study

In order to appropriately frame the incidence data presented in this mode-of-action experiment, the findings presented here were compared to the guideline definitive developmental toxicity study in Crl:CD(SD) rats. Treatment-related findings in the offspring were found at 1000 ppm in the developmental toxicity study and in Group 3 of this study. Despite the fact that Group 2 dams in this study were exposed at a sufficient dose (76.5 mg/kg/day) to induce an effect as seen at 1000 ppm in the developmental toxicity study, they were negative for treatment-related

offspring effects. A comparison of results from the developmental toxicity study and the current study is presented below.

Table 4.11.3.1.Study 4.13 (DAR Table B.6.6.12.4-13): Comparison to Developmental Toxicity Study

	Developmental Toxicity Study (MRID 47832140)			**Critical Window 1 (current study)**	
	Group 2	**Group 3**	**Group 4**	**Group 2**	**Group 3**
Dose (PPM)	25	150	1000	1000	1000
Treatment period	GD 6-21	GD 6-21	GD 6-21	GD 6-16	GD 16-Birth
Avg.TMI (mg/kg/day)	1.95	11.5	70.2	76.5	38.6
TK plasma conc. (µg/g)	D 0.843 +/- 0.09 F 0.644 +/- 0.07	D 4.938 +/- 0.87 F 4.065 +/- 0.64	D 35.245 +/- 5.43 F 30.001 +/- 5.25	35.4 - 40.9	32.1 - 43.2
Offspring Effects	NO	NO	**YES**	NO	**YES**

In order to provide a quantitative comparison of offspring effects, incidence data from the two affected groups in Table B.6.6.12.4-13 were analysed and comparisons made to the applied and systemic sulfoxaflor doses in these groups.

Table 4.11.3.1.Study 4.14 (DAR Table B.6.6.12.4-13.): Comparison of Affected Groups

	Group 4	**Group 3**
Dose (PPM)	1000	1000
Treatment period	GD 6-21	GD 16-Birth
Avg.TMI (mg/kg/day)	70.2	38.6
TK plasma conc. (µg/g)	D 35.245 +/- 5.43 F 30.001 +/- 5.25	32.1 - 43.2
Pup death	N/A	53.1%
Forelimb Flexure[1]	F 122/295 (41.4) L 23/24 (95.8)	P 50/143 (35.0) [2] L 11/12 (91.7) [2]
Hindlimb Rotation	F 12/295 (4.1) L 7/24 (29.2)	P 19/143 (13.3) [2] L 8/12 (66.7) [2]
Convoluted Ureter	F 19/149 (12.8) L 7/24 (29.2)	P 0/49 (0)[3] L 0/7 (0)[3]
Bent Clavicle	F 40/133 (30.1) L 17/24 (70.8)	P 0/49 (0)[3] L 0/7 (0)[3]

D = Dam, F = foetus, P = pup, L = litter

[1]A severe, >90º, persistent flexure at the wrist or any flexure which cannot straighten.

[2]Evaluated in surviving pups on PND 0.
[3]Evaluated in surviving pups on PND 4

Maternal sulfoxaflor plasma concentrations were similar between Group 4 dams in the developmental toxicity study (35.24 ± 5.43 µg sulfoxaflor/g plasma) and Group 3 dams in the current study (32.1-43.2 µg sulfoxaflor/g plasma). Correlating with these blood levels was a similar incidence of forelimb flexure (a severe, >90º, persistent flexure at the wrist or any flexure which cannot straighten) on a foetal/pup or litter basis. Unlike the developmental toxicity study, which had timed evaluations of GD 21 foetuses, this critical window study observed pups at different times after birth where subtle observations, such as slight forelimb flexure (a 45-90º bend that can be straightened with movement), would vary over time and could be confounded by postnatal limb movements. Therefore, the comparison table (Table B.6.6.12.4-13) includes indices for forelimb flexure, but excludes the slight forelimb flexure incidence data from the developmental toxicity study.

The percent incidence of hindlimb rotation was higher in the current study, which is attributed to normal biological variability and/or timing of examination (GD 21 foetuses *vs*. postnatal pups). It is important to note that no surviving PND 4 offspring had limb abnormalities, nor did they have convoluted ureters or bent clavicle. Given the similarities in incidence of the limb abnormalities and bent clavicles, it is very likely that offspring in this treatment group had these findings at the time of birth, which subsequently resolved.

Conclusions

The critical window of susceptibility in rats for the foetal abnormalities of limb contractures and reduced neonatal survival resulting from maternal exposure to 1000 ppm sulfoxaflor *via* the diet falls within the exposure period of GD16-birth. These abnormalities are reversible upon birth upon withdrawal of maternal dietary exposure. These results support the hypothesis that late gestational exposure to sulfoxaflor induces foetal abnormalities and neonatal death *via* its pharmacological action on the foetal muscle nicotinic acetylcholine receptor (nAChR), which develops functional expression during this stage of gestation.

Study 5: Critical window Phase 2

This was the second of two studies conducted to determine the critical window of susceptibility, and to test the hypothesis that late gestational exposure to sulfoxaflor induces foetal abnormalities and reduced neonatal survival *via* its pharmacological action on the foetal muscle nicotinic acetylcholine receptor (nAChR). This receptor develops functional expression between GD 16 and 17 in the rat, resulting in synchronised foetal limb movements and diaphragmatic responsiveness important for the transition to extrauterine respiration.

The purpose of this study was to further refine the critical window of sulfoxaflor exposure that is sufficient to cause foetal abnormalities and reduce neonatal survival. This study divided the GD 16-birth exposure window - shown in the first study to be the exposure period responsible for both effects - into three 48-hour exposure windows starting on the morning of GD 16, 18, or 20. Groups of 10 female Crl:CD(SD) rats were administered control diet (Group 1), or diets containing 1000 ppm sulfoxaflor (the high dose level from the developmental toxicity study) fed

from GD 16-18 (Group 2), GD 18-20 (Group 3), or GD 20-22 (Group 4). In the offspring, effects on litter size, survival, body weight and the presence of gross external morphological alterations, with particular focus on limb abnormalities (e.g., forelimb flexure and hindlimb rotation), were carefully assessed. In addition, a subset of animals was examined for the presence of convoluted ureters and bent clavicles as these effects had also been seen in the sulfoxaflor rat developmental toxicity study at 1000 ppm

Offspring from animals given 1000 ppm sulfoxaflor for 48 hours starting on the morning of GD 16 or 18 (Group 2 and 3) were similar to controls and did not display previously described foetal abnormalities or reduced neonatal survival. In contrast, offspring given 1000 ppm sulfoxaflor for 48 hours starting on the morning of GD 20 (Group 4) had foetal limb abnormalities (forelimb flexure and hindlimb rotation) as well as reduced neonatal survival, demonstrating that exposure shortly before birth (GD 21 or 22) is sufficient to induce developmental toxicity.

In addition, daily examination of surviving Group 4 offspring born with limb abnormalities indicated that these were fully reversible in surviving offspring shortly after withdrawal of maternal dietary exposure to sulfoxaflor. In some cases, full reversal of the limb abnormalities was evident the day after birth and occurred for all affected animals that survived to postnatal day (PND) 4; reversal also occurred in some animals that subsequently died before PND 4. Likewise, the visceral and skeletal findings of abnormal ureter and bent clavicle, the latter of which had a high incidence (30.1% of foetuses), in the definitive developmental toxicity study were not present in this study at necropsy on PND 4.

In summary, this study demonstrated that the critical period of developmental susceptibility to sulfoxaflor-induced foetal abnormalities and reduced neonatal survival effects occurs shortly before birth, and that the foetal abnormalities are rapidly reversible after birth. These results support the hypothesis that late gestational exposure to sulfoxaflor induces foetal abnormalities and neonatal death *via* its pharmacological action on the foetal muscle nicotinic acetylcholine receptor (nAChR), which develops functional expression during this stage of gestation. This study non-guideline study is considered acceptable.

Report:	XDE 208: Investigation of the critical window of exposure for fetal abnormalities and neonatal survival effects in Crl:CD(SD) rats (Phase 2).
Author:	Rasoulpour, R. and C. Zablotny
Date of Report:	**2**4 June, 2010
Report Identity:	Study ID: 091049
Testing Facility:	Toxicology & Environmental Research and Consulting, The Dow Chemical Company (Michigan).
GLP	Signed and dated GLP (non-compliance), Quality Assurance, and (No) Data Confidentiality statements were provided. .
Test Substance:	XDE-208 (95.6% (wt/wt); as two diastereomers in 48.4/47.4% ratio.
Batch:	E2162-34
Guidelines:	Non-guideline
Deviations:	Not applicable
Acceptable:	Yes

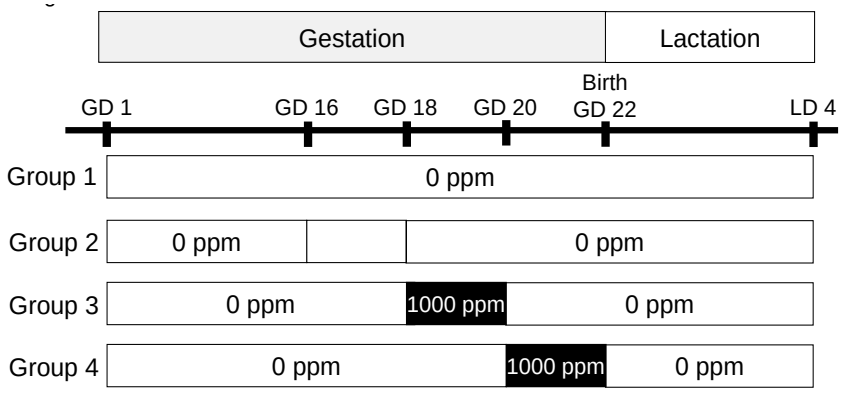
Materials and Methods

The purpose of this study was to further refine the critical window of sulfoxaflor exposure that is

sufficient to cause foetal abnormalities and reduce neonatal survival. This study divided the GD 16-birth exposure window – (shown in the first study to be the exposure period responsible for both effects)- into three 48-hour exposure windows starting on the morning of GD 16, 18, or 20. Groups of 10 female Crl:CD(SD) rats were administered control diet (Group 1), or diets containing 1000 ppm sulfoxaflor (the high dose level from the developmental toxicity study) fed from GD 16-18 (Group 2), GD 18-20 (Group 3), or GD 20-22 (Group 4), corresponding to mean intakes of 0, 63.9, 42.5, and 35.7 mg/kg/day for Groups 1, 2, 3, and 4 respectively. In the offspring, effects on litter size, survival, body weight and the presence of gross external morphological alterations, with particular focus on limb abnormalities (e.g., forelimb flexure and hindlimb rotation), were carefully assessed. In addition, a subset of animals was examined for the presence of convoluted ureters and bent clavicles as these effects had also been seen in the sulfoxaflor rat developmental toxicity study at 1000 ppm

Study schedule: : Groups of 10 time-mated female Crl:CD(SD) rats were administered diets containing either 0 or 1000 ppm sulfoxaflor for 48 hours beginning on GD 16, 18, or 20 (Figure B.6.6.12.5-1).

Figure 4.11.3.1.Study 5.1 (DAR Figure B.6.6.12.5-1)



The key study parameters and study schedule are outlined in Table B.6.6.12.5.-2.

Table 4.11.3.1.Study 5.1 (DAR Table B.6.6.12.5-1.): Summary of Key Study Events and Parameters

Study Events and Parameters	No. Animals[1]	Timing
Cage-side examinations	All[2]	At least twice daily
Clinical observations	All	GD 1, 6, 9, 14, 16, 18 and 20, 22; LD 0-4
Maternal body weights	All	GD 0 by the supplier, on GD 6, 9, 14, daily from GD 6-22, and on LD 1 and 4 for females delivering a litter
Feed consumption	All	On GD 6-9, 9-14, daily from GD 16-22, and on LD 1 and 4 for females delivering a litter
Maternal blood collection	4/group	On GD 18 for groups 1 and 2, on GD 20 for groups 1 and 3, and on GD 22 for groups 1 and 4
Litter observations	All	Daily from PND 0-4
No. of live & dead pups	All	PND 0-4
Pup Body Weights	All	PND 1 and 4
Termination of pups	All	PND 4
Convoluted ureter and bent clavicle examination	Group 1 and 4	PND 4
Termination of adult females	All	LD 4 or at least 24 days after evidence of mating for females not delivering a litter

[1]Adults unless specified otherwise;
[2]includes dams and their litters;
GD = gestation day, LD = lactation day, PND = postnatal day,
N/A = not applicable

Results:

Dietary analysis: Analyses of the 1000 ppm diet, plus control and premix, revealed concentrations ranging from 97.1% to 98.3% of the targeted concentration. Analysis of aliquots of the 1000 ppm diet indicated that the test material was homogeneously distributed, based on a relative standard deviation (RSD) of 1.9%. A previously conducted stability study showed sulfoxaflor to be stable for at least 65 days in rodent feed at concentrations ranging from 0.0005 to 10%.

Maternal observations

Mortality/clinical; signs: All maternal animals survived until termination. No treatment-related effects on behaviour or demeanour were observed at any dose level during the gestation or lactation period. There were no notable observations made during the cage-side observations.

Body weight: Dams given 1000 ppm sulfoxaflor (Groups 2-4) had treatment-related effects on body weight gain during their respective treatment intervals. These body weight effects were attributed to lower feed consumption due to decreased palatability of the test material fortified diet and consistent with effects at 1000 ppm in the definitive developmental toxicity study. Mean maternal body weight gains during gestation are summarized in Table B.6.6.12.5-2.

Table 4.11.3.1.Study 5.2 (DAR Table B.6.6.12.5-2)

Table 3. Maternal Gestation Body Weight Gains[a]										
Group No./ Treatment Period (n)	**Mean Body Weight Gain (grams±SD)**									
	GD 0-6	**GD 6-9**	**GD 9-14**	**GD 14-16**	**GD 16-17**	**GD 17-18**	**GD 18-19**	**GD 19-20**	**GD 20-21**	**GD 0-21**
1 N/A - Control (10)	40.6 ±5.6	17.0 ±4.2	28.2 ±3.8	23.1 ±5.3	3.6 ±3.6	16.4 ±8.6	9.3 ±6.2	12.6 ±5.6	8.4 ±5.6	159.2 ±18.6
2 GD 16-18 (9)	34.2 ±7.8	18.3 ±4.7	27.8 ±6.9	24.1 ±3.1	-4.1* ±2.9	15.6 ±6.2	14.7 ±8.7	14.1 ±4.4	12.1 ±5.7	157.0 ±26.5
3 GD 18-20 (9)	36.8 ±6.7	15.7 ±3.7	26.3 ±8.5	25.0 ±4.7	4.1 4.2±	19.9 ±4.6	0.1* ±4.2	5.4* ±7.4	15.5 ±8.9	148.9 ±18.8
4 GD 20-22/LD 0 (8)	35.3 ±9.9	19.3 ±5.5	24.6 ±7.1	25.2 ±4.3	3.8 ±5.8	20.1 ±5.2	8.4 ±3.8	14.3 ±4.9	3.1 ±3.7	154.1 ±24.8

Food consumption: Animals given 1000 ppm sulfoxaflor (Groups 2-4) had treatment-related decreases in feed consumption during their respective treatment intervals. These findings were consistent with decreased palatability of test material fortified diet observed in previous studies at this concentration.

Table 4.11.3.1.Study 5.3 (DAR Table B.6.6.12.5-3): Feed Consumption during Gestation[a]

Group No./ Treatment Period (n)	**Mean feed consumption (grams/day±SD)**								
	GD 6-9	**GD 9-14**	**GD 14-16**	**GD 16-17**	**GD 17-18**	**GD 18-19**	**GD 19-20**	**GD 20-21**	**GD 21-22[b]**
1 NA – Control (10)	21.2 ±2.2	23.0 ±2.5	23.4 ±2.3	25.1 ±4.2	23.2 ±5.7	18.9 ±2.7	21.1 ±2.2	19.6 ±3.3	11.7 ±6.1
2 GD 16-18 (9)	20.6 ±2.1	22.4 ±2.1	23.4 ±2.0	21.1* ±2.6	19.1 ±3.3	18.6 ±3.7	22.6 ±3.0	21.5 ±4.8	13.1 ±6.3

Group No./ Treatment Period (n)	Mean feed consumption (grams/day±SD)								
	GD 6-9	GD 9-14	GD 14-16	GD 16-17	GD 17-18	GD 18-19	GD 19-20	GD 20-21	GD 21-22[b]
3 GD 18-20 (9)	20.1 ±1.7	22.2 ±2.9	24.8 ±3.0	26.9 ±2.2	25.7 ±2.7	14.3* ±1.5	14.2* ±4.7	20.1 ±5.0	13.3 ±4.8
4 GD 20-22/LD 0 (8)	20.3 ±2.3	22.1 ±2.3	23.2 ±3.1	26.5 ±3.3	25.2 ±3.3	19.0 ±2.3	21.9 ±2.5	14.1* ±2.0	2.9* ±2.4

Test substance uptake: Time-weighted average doses for groups 2-4 were 63.9, 42.5, and 35.7 mg/kg/day, respectively. The decrease in dose across these groups is attributed to the increase in dam body weight near the end of pregnancy associated with foetal growth and lower feed consumption near parturition.

Toxicokinetic data: There was no sulfoxaflor found in plasma obtained from dams of the control group. Blood samples were taken from dams in the treated groups at the end of their 48-hour treatment interval. The measured plasma concentrations of Groups 2, 3, and 4 ranged from 16.4 33.3, 23.0-30.2, and 5.41-16.1 µg sulfoxaflor /g plasma, respectively. The plasma concentration of sulfoxaflor in Group 4 was lower than the other groups because three of the four sampled rats had undergone parturition; therefore, feed consumption, and corresponding test material intake, in these animals was minimal. The sulfoxaflor plasma concentration from the one Group 4 animal that had not given birth (#1640) was 16.1 µg/g plasma. The mean plasma concentrations of the control and test substance treatment groups are summarized in Table B.6.6.12.5-2.

Table 4.11.3.1.Study 5.4 (DAR Table B.6.6. 12.5-4) Toxicokinetic Data[a]

Gestation/ Lactation Day	Measured Plasma Concentration (µg sulfoxaflor/g plasma)			
	Group No./Treatment Period (n)			
	1 Control (3)	2 GD 16-18 (4)	3 GD 18-20 (4)	4 GD 20-22/LD 0 (4)
GD 18	<LLQ	16.4-33.3	N/A	N/A
GD 20	<LLQ	N/A	23.0-30.2	N/A
GD 22	<LLQ	N/A	N/A	16.1
LD 0	<LLQ	N/A	N/A	5.41-6.69

N/A – not applicable
<LLQ = below the lowest limit of quantitation (0.137 µg sulfoxaflor/g plasma)

Litter effects

Pup survival/sex ratio: There were no treatment-related effects on gestation survival or sex ratios. Administration of 1000 ppm sulfoxaflor from GD 16-birth had previously been shown to decrease pup survival between PND 0-4 (First Critical Window Study). In this study, treatment-related effects on pup survival were limited to Group 4 litters that were exposed to 1000 ppm sulfoxaflor for 48 hours starting on the morning of GD 20 (89.6% *vs* 99.1% in controls). It is also important to note that there was no effect on pup survival in Groups 2 or 3, which were administered 1000 ppm sulfoxaflor for 48 hours starting on the mornings of GD 16 or 18, respectively. These data demonstrate that a very short duration of exposure (i.e., one or two days) before birth was sufficient to decrease pup survival, supporting the hypothesis that these developmental toxicity effects are pharmacologically based and consistent with action on the rat foetal nAChR.

Normalised for the lower systemic exposure (5.41-16.1 µg sulfoxaflor/g plasma) the pup survival incidence observed with Group 4 in this study is consistent with the previous OECD 421-like reproduction/developmental toxicity screening study and the first critical window study. Blood levels were not measured in the 500 ppm group of the OECD 421-like study, but for comparison purposes measured blood levels at 400 ppm in the two-generation reproductive toxicity study were 14.9-16.6 µg/g plasma and PND 4 pup survival was 95.4 and 95.5% for the first and second generations, respectively. These data demonstrate that when normalised to systemic exposure (i.e., blood concentrations), a longer duration of exposure did not result in a more robust effect than GD 20-22/LD 0. A comparison of pup survival in the current study to pup survival in the OECD-like study and the critical window 1 study is presented in Table B6.6.12.5-3.

Table 4.11.3.1.Study 5.5 (DAR Table B.6.6.12.5-3.): Pup Survival Comparison

	OECD 421-like study(MRID 47832143)			**Critical Window 1**	**Critical Window 2 (current study)**
Dose (ppm):	**100**	**500**	**1000**	**Group 3 1000**	**Group 4 1000**
Treatment period:	**Two weeks prior to breeding - PND 21**			**GD 16-brith**	**GD 20-22/LD 0**
Test Material Intake (mg/kg/day)	GD 14-20 6.07	GD 14-20 30.3	GD 14-20 62.0	GD 16-birth 38.6	GD 20-22/LD 0 35.7
TK plasma conc (µg SULFOXAFLOR/g)	N/A[a]	N/A[b]	LD4 14.3 - 41.9	GD21 32.1 - 43.2	5.41 - 16.1[c]
PND 4 pup survival	97.8%	**81.2%**	**7.3%**	**46.9%**	**89.6%**
Pup death	2.2%	**18.8%**	**92.7%**	**53.1%**	**10.4%**

Results **in bold type** indicate the effects judged to be treatment-related.
[a, b] = TK data not collected, but for comparison LD4 blood levels from two-generation reproduction study were 3.84-5.15 µg/g plasma at 100 ppm and 14.9-16.6 µg/g plasma at 400 ppm
[d] = Three of the four sampled rats had undergone parturition prior to sampling

Litter size/mean body weight: : There were no clear treatment-related differences in the number of pups born alive or dead, pup body weight, or litter size in Groups 2-4 when compared to controls (Group 1). Consistent with effects on pup survival, Group 4 mean litter size was decreased from 11.9 to 10.8 pups between PND 1-2. Data for pups born alive/dead and litter size are summarised in Table B6.6.12.5-4. Data for pup body weight are summarised in Table B.6.6.12.5-5.

Table 4.11.3.1.Study 5.6 (DAR Table B.6.6.12.5-4.): Litter Size

	Group no/treatment period (n)			
	1 NA -Control (10)	**2 GD 16-18 (9)**	**3 GD 18-20 (9)**	**4 GD 20-22/LD 0 (8)**
Born live	11.4±1.2	11.0±2.2	11.7±1.8	12.0±1.6
Born dead	0.0±0.0	0.1±0.3	0.0±0.0	0.0±0.0
	Mean Litter Size			
LD 1	11.4±1.2	10.9±2.3	11.6±1.9	11.9±1.6
LD 2	11.4±1.2	10.9±2.3	11.6±1.9	10.8±3.2
LD 3	11.4±1.2	10.9±2.3	11.6±1.9	10.8±3.2
LD 4	11.3±1.3	10.9±2.3	11.6±1.9	10.9±3.2

Table 4.11.3.1.Study 5.7 (DAR Table B.6.6.12.5-5): Pup Body Weights

Lactation Day	**Mean Body Weight (grams±SD)**							
	Group No./Treatment Period (n)							
	1 NA - Control (10)		**2 GD 16-18 (9)**		**3 GD 18-20 (9)**		**4 GD 20-22/LD 0 (8)**	
	F	**M**	**F**	**M**	**F**	**M**	**F**	**M**
LD 1	6.8 ±0.8	7.2 ±0.8	6.8 ±0.4	7.1 ±0.5	6.6 ±0.4	6.9 ±0.5	6.3 ±0.7	6.7 ±0.6
LD 4	10.0 ±1.0	10.4 ±1.0	10.0 ±0.4	10.5 ±0.5	9.5 ±0.7	9.9 ±0.7	9.3 ±0.8	9.7 ±0.8

M = male, F = female

Pup anatomical alterations: Offspring from dams given 1000 ppm sulfoxaflor for 48 hours starting on the morning of GD 20 (Group 4) were observed with the limb abnormalities of

forelimb flexure (<90º) and hindlimb rotation, as previously identified in the definitive developmental toxicity study in rats given 1000 ppm from GD 6-21 and from GD 16-birth in the first critical window study. On the day of birth (PND 0) 7.3% of pups from 50.0% of litters were observed with forelimb flexure, while 11.5% of pups from 75.0% of litters were observed with hindlimb rotation. This incidence of limb abnormalities decreased over the subsequent 48 hours due to a combination of pup death and reversibility of the limb abnormalities (see Reversibility section), as evidenced by a 0% incidence of forelimb flexure and hindlimb rotation by PND 2. Consistent with the reduced neonatal survival and limb abnormality effects, only 1 of 6 Group 4 dams had a litter within normal limits compared to 5 of 10, 8 of 9, and 6 of 9 in Groups 1, 2, and 3 respectively. Similar to the pup survival effects, limb alterations observed in the offspring from Groups 2 and 3 were unaffected. The limb alterations observed in Group 4 are summarised in Table B.6.6.12.5-6.

Table 4.11.3.1.Study 5.8 (DAR Table B.6.6.12.5-6.): Limb Alterations Observed in Group 4

Postnatal Day	Forelimb Flexure No. Affected/Total No. (percent)		Hindlimb Rotation No. Affected/Total No. (percent)	
	Pups	Litter	Pups	Litter
PND 0	7/96 (7.3%)	4/8 (50.0%)[a]	11/96 (11.5%)	6/8 (75.0%)[a]
PND 1	4/95 (4.2)	3/8 (37.5)	5/95 (5.3)	2/8 (25.0)
PND 2	0/86 (0.0)	0/8 (0.0)	0/86 (0.0)	0/8 (0.0)
PND 3	0/86 (0.0)	0/8 (0.0)	0/86 (0.0)	0/8 (0.0)
PND 4	0/86 (0.0)	0/8 (0.0)	0/86 (0.0)	0/8 (0.0)

[a] Statistically different from the control ($p<0.05$) using censored Wilcoxon's test.

On PND 4, all available pups/sex/litter from Groups 1 and 4 underwent visceral examination to determine the presence or absence of convoluted ureters and skeletal examination for bent clavicles, which were identified as treatment-related effects in the definitive developmental toxicity study at 1000 ppm sulfoxaflor. There were no observations of convoluted ureters or bent clavicles in the 113 pups evaluated in Group 1. Despite the fact that offspring in Group 4 exhibited treatment-related limb contractures (forelimb flexure and hindlimb rotation) and pup death, there were no observations of convoluted ureters or bent clavicles in the 86 pups evaluated from this group.

Reversibility: As mentioned above, offspring from dams given 1000 ppm sulfoxaflor for 48 hours starting on the morning of GD 20 (Group 4) were observed on the day of birth (PND 0) with forelimb flexure (7.3% of pups from 50.0% of litters) and hindlimb rotation (11.5% of pups from 75.0% of litters); however, by PND 2 there were no incidences of either limb abnormality. This was consistent with the reversals seen in the first critical window study. Data from this study were added to the reversal table in that study, which tracks the fate of each litter with respect to the number of pups alive, number of pups dead, and number of pups with limb abnormalities on each day. As seen in the first critical window study, it became apparent that the limb abnormalities must have reversed in some pups. For example, dam 1637 gave birth to 11 live pups of which one had limb abnormalities on PND 0. On the next day, there were still 11 live pups, but none had limb abnormalities indicating one reversal between PND 0 and 1. In this study, seven pups with limb abnormalities could be tracked with certainty demonstrating reversal between PND 0 and 2. Adding the data from the first critical window study, there were a total of 28 confirmed limb abnormality reversals in 11 of 20 total litters from these two studies, showing

clear reversibility of limb abnormalities. These data suggest that the limb abnormalities were transient, consistent with a pharmacologic mode-of-action for sulfoxaflor. A comparison of the limb reversal data for the current study and the Critical Window 1 study is presented in Table B.6.6.12.5-7.

Table 4.11.3.1.Study 5.9 (DAR Table B.6.6.12.5-7.): Limb Abnormality Reversals (pups/day)

Post-natal Day	Critical Window 2[a] (current study)				Critical Window 1
	Group No./Treatment Period				
	Group 1 Control	**Group 2** GD 16-18	**Group 3** GD 18-20	**Group 4** GD 20-22/LD 0	**Group 3** GD 16-birth
PND 0 to 1	N/A	N/A	N/A	5	11
PND 1 to 2	N/A	N/A	N/A	2	6
PND 2 to 3	N/A	N/A	N/A	0	3
PND 3 to 4	N/A	N/A	N/A	0	1
Total Reversals	N/A	N/A	N/A	**7**	**21**

N/A – not applicable

In addition to reversal of the limb abnormalities there were no observations of convoluted/hydroureter ureters or bent clavicles at PND 4, despite the fact that these findings were observed on GD 21 foetuses from dams exposed to 1000 ppm sulfoxaflor in the developmental toxicity study, and at a relatively high incidence (30.1% of foetuses) for bent clavicles. Foetal convoluted/hydroureter are variants that result from a temporary build-up of urine in the ureter and are known to be readily reversible after birth. Postnatal remodelling of skeletal abnormalities has been shown with other test compound such as caffeine and ethylene glycol. This provides further evidence of reversibility in that convoluted ureter and bent clavicles also appear to have the ability to reverse during the early postnatal period, which suggests that they are also transient alterations consistent with a pharmacologic mode of action of sulfoxaflor.

Comparison to Developmental toxicity study: In order to appropriately frame the incidence data presented in this mode-of-action experiment, the findings presented here were compared to the guideline definitive developmental toxicity study in Crl:CD(SD) rats and the first critical window study. Treatment-related findings in the offspring were found at 1000 ppm in the

developmental toxicity study Group 3 in critical window 1 (1000 ppm sulfoxaflor from GD 16-birth) and in Group 4 of this study. Despite the fact that Group 2 and 3 dams in this study were exposed at a sufficient dose (62.9 and 42.5 mg/kg/day, respectively) to induce an effect as seen at 1000 ppm in the developmental toxicity study, they were negative for treatment-related offspring effects. A comparison of results from the developmental toxicity study, the critical window 1 study and the current study is presented in Table B.6.6.12.5-8.

Table 4.11.3.1.Study 5.9 (DAR Table B.6.6.12.5-8.): Comparison of critical window studies to the Developmental Toxicity Study

	Developmental Toxicity Study(MRID 47832140)			**Critical Window 1 (MRIF 47832137)**		**Critical Window 2 (current study)**		
Group:	**Group 2**	**Group 3**	**Group 4**	**Group 2**	**Group 3**	**Group 2**	**Group 3**	**Group 4**
Dose (ppm):	**25**	**150**	**1000**	**1000**	**1000**	**1000**	**1000**	**1000**
Treatment period:	**GD 6-21**	**GD 6-21**	**GD 6-21**	**GD 6-16**	**GD 16-Birth**	**GD 16-18**	**GD 18-20**	**GD 20-22/LD 0**
Avg.TMI (mg/kg/day)	1.95	11.5	70.2	76.5	38.6	62.9	42.5	35.7
TK plasma conc. (µg/g)	0.843 ± 0.09	4.938 ± 0.87	35.245 ± 5.43	35.4 – 40.9	32.1 - 43.2	16.4 - 33.3	23.0 - 30.2	5.41 - 16.1[a]
Offspring Effects	NO	NO	**YES**	NO	**YES**	NO	NO	**YES**

GD = gestation day, LD = lactation day

[a] = Three of the four sampled rats had undergone parturition prior to blood collection

In order to provide a quantitative comparison of offspring effects, incidence data from the three affected groups in Table B.6.6.12.5-8 were analysed and comparisons made to the applied and systemic sulfoxaflor doses in these groups. The results of the comparison are presented in Table B.6.6.12.5-9.

Table 4.11.3.1.Study 5.10 (DAR Table B.6.6.12.5-9.): Comparison of Affected Groups

	Developmental Toxicity Study	**Critical Window 1**	**Critical Window 2 (current study)**
Group:	**Group 4**	**Group 3**	**Group 4**
Dose (ppm):	**1000**	**1000**	**1000**
Treatment period:	**GD 6-21**	**GD 16-Birth**	**GD 20-22/LD0**
Avg.TMI (mg/kg/day)	70.2	38.6	35.7
TK plasma conc. (µg/g)	D 35.245 +/- 5.43 F 30.001 +/- 5.25	D 32.1 - 43.2	D 5.41 - 16.1[a]
Pup death	N/A	53.1%	10.4%
Forelimb Flexure[b] No. affected/Total No. (%)	F 122/295 (41.4) L 23/24 (95.8)	P 51/143 (35.7)[c] L 11/12 (91.7)[c]	P 7/96 (7.3)[c] L 4/8 (50.0)[c]
Hindlimb Rotation No. affected/Total No. (%)	F 12/295 (4.1) L 7/24 (29.2)	P 19/143 (13.3)[c] L 8/12 (66.7)[c]	P 11/96 (11.5)[c] L 6/8 (75.0)[c]
Convoluted Ureter No. affected/Total No. (%)	F 19/149 (12.8) L 7/24 (29.2)	P 0/49 (0)[d] L 0/12 (0)[d]	P 0/99 (0)[d] L 0/9 (0)[d]
Bent Clavicle No. affected/Total No. (%)	F 40/133 (30.1) L 17/24 (70.8)	P 0/49 (0)[d] L 0/12 (0)[d]	P 0/99 (0)[d] L 0/9 (0)[d]

D=dam,F=foetus,P=pup,L=litter
N/A = not applicable
[a] Three of the four sampled rats had undergone parturition prior to blood collection
[b] A severe, >90º, persistent flexure at the wrist or any flexure which cannot straighten
[c] Evaluated in surviving pups on PND 0.
[e] Evaluated in surviving pups on PND 4.

Although the applied dose was similar between Group 3 of critical window 1 (38.6 mg/kg/day) and Group 4 of the current study (35.7 mg/kg/day), indices of pup death and forelimb flexure (a severe, >90º, persistent flexure at the wrist or any flexure which cannot straighten) were lower. This is attributed to the fact that rats give birth on GD 21 or 22 and by starting exposure on the

morning of GD 20, many animals were exposed to test material for only one day and had not yet achieved steady state blood levels. This is confirmed by the lower measured systemic dose (5.41-16.1 µg sulfoxaflor/g plasma) in these animals from Group 4 of the current study.

As mentioned in the first critical window study, unlike the developmental toxicity study, which had timed evaluations of GD 21 foetuses, the critical window studies observed pups at different times after birth where subtle observations, such as slight forelimb flexure (a 45-90º bend that can be straightened with movement), would vary over time and could be confounded by postnatal limb movements. Therefore, the comparison table (Table 14) includes indices for forelimb flexure but excludes the slight forelimb flexure incidence data from the developmental toxicity study.

Unlike forelimb flexure, the percent incidence of hindlimb rotation was similar in the current study to the first critical window study and higher than the developmental toxicity study. This is attributed to normal biological variability and/or timing of examination (GD 21 foetuses vs. postnatal pups). It is important to note that no surviving PND 4 offspring had limb abnormalities, nor did they have convoluted ureters or bent clavicle. Given the similarities in incidence of the limb abnormalities and bent clavicles, it is very likely that offspring in this treatment group had these findings at the time of birth, which subsequently resolved.

Conclusions

Offspring from animals given 1000 ppm sulfoxaflor for 48 hours starting on the morning of GD 16 or 18 (Group 2 and 3) were similar to controls and did not display previously described foetal abnormalities or reduced neonatal survival. In contrast, offspring given 1000 ppm sulfoxaflor for 48 hours starting on the morning of GD 20 (Group 4) had foetal limb abnormalities (forelimb flexure and hindlimb rotation) as well as reduced neonatal survival, demonstrating that exposure shortly before birth (GD 21 or 22) is sufficient to induce developmental toxicity.

This study demonstrated that the critical period of developmental susceptibility to sulfoxaflor-induced foetal abnormalities and reduced neonatal survival effects occurs shortly before birth (GD 21 or 22), and that the foetal abnormalities are rapidly reversible after birth. These results support the hypothesis that late gestational exposure to sulfoxaflor induces foetal abnormalities and neonatal death *via* its pharmacological action on the foetal muscle nicotinic acetylcholine receptor (nAChR), which develops functional expression during this stage of gestation

Study 6: Diaphragm contracture.

Sulfoxaflor, a compound targeted to the insect nicotinic acetylcholine receptor (nAChR) has been shown to cause foetal limb contractions and reduced neonatal survival in rats following dietary exposure during gestation. It is hypothesised that these effects result from activation of the foetal muscle-type nAChR bysulfoxaflor, thereby causing sustained muscle contracture in the foetus and inhibition of nerve-evoked contraction of the diaphragm that would cause impaired respiration after birth resulting in the previously observed reductions in neonatal survival. In support of this hypothesis, sulfoxaflor has been demonstrated to be an agonist at rat, but not

human, embryonic ((α1)2β1δγ)) nAChR, while being without agonist activity at mature ((α1)2β1δε)) muscle–type nAChRs (rat or human). The aim of the work described in this report was to make a qualitative investigation of the action of sulfoxaflor on isolated phrenic nerve-hemidiaphragm preparations from new-born rats. Sulfoxaflor consistently (n=5) produced a reversible, concentration-dependent contracture of the diaphragm that was blocked by the selective muscle-type nAChR antagonist, tubocurarine (10 µM) showing that the contracture induced by sulfoxaflor is mediated *via* nAChR activation, rather than *via* a post-receptor mechanism. Furthermore, prolonged application of sulfoxaflor caused a sustained muscle contracture. Muscle twitches in response to phrenic nerve stimulation were not affected at low sulfoxaflor concentration (100 µM) but were reduced at high concentration (1 mM) demonstrating that sulfoxaflor can cause inhibition of nerve-evoked contraction of the diaphragm during sustained contracture, consistent with the observed impairment of respiration in the neonatal rat. The results of these experiments demonstrate that sulfoxaflor caused a contracture of the new-born rat diaphragm by acting on the nAChR. Prolonged application caused a sustained muscle contracture and a contracture-associated inhibition of the phrenic nerve-evoked muscle twitch, which is considered analogous to the situation *in vivo* which resulted in foetal limb contractions (sustained muscle contractions) and compromised respiration at birth (contracture-associated inhibition of the muscle twitch). Therefore, the results described in this report are entirely consistent with, and add additional support to, the hypothesis that sulfoxaflor causes neonatal death (and foetal abnormalities) *via* activation of the foetal muscle-type nAChR.

Report:	Observations on the effects of XDE-208 on the phrenic nerve-hemidiaphragm preparation from new-born rat.
Author:	Alasdair J. Gibb, Ph.D. (2010).
Date of Report:	30 June, 2010
Report Identity:	UCL Diaphragm (30 June 2010).
Testing Facility:	Research Department of Neuroscience, Physiology & Pharmacology, University College London, Gower Street, London, WC1E 6BT, United Kingdom.
GLP	Signed and dated Data Confidentiality statements were not provided. .
Test Substance:	XDE-208 (95.6% (wt/wt); as two diastereomers in 50/49.5% ratio.
Batch:	E2162-34
Guidelines:	Non-guideline
Deviations:	Not applicable
Acceptable:	Yes

Introduction

Sulfoxaflor, a compound targeted to the insect nicotinic acetylcholine receptor (nAChR) has been shown to cause foetal limb contractions and reduced neonatal survival in rats following dietary exposure during gestation. It is hypothesised that these effects result from activation of the foetal muscle-type nAChR bysulfoxaflor, thereby causing sustained muscle contracture in the foetus and inhibition of nerve-evoked contraction of the diaphragm that would cause impaired respiration after birth resulting in the previously observed reductions in neonatal survival. In support of this hypothesis, sulfoxaflor has been demonstrated to be an agonist at rat, but not human, embryonic ((α1)2β1δγ)) nAChR, while being without agonist activity at mature ((α1)2β1δε)) muscle–type nAChRs (rat or human). The aim of the work described in this report

was to make a qualitative investigation of the action of sulfoxaflor on isolated phrenic nerve-hemidiaphragm preparations from new-born rats.

Materials and Methods

Isolated phrenic nerve-hemidiaphragm preparations from new-born rats were used. All experiments were carried out in accordance with the Animals (Scientific Procedures) Act 1986 and in accordance with local ethical approvals. Preparations were constantly superfused at 1.5 mL min-1 in a total bath volume of 0.5 mL with physiological salt solution containing (in mM): NaCl (125), KCl (3), NaHCO3, (25) NaH2PO4 (1.0), CaCl2 (2.5), MgCl2 (1.0), and glucose (25) at pH 7.4 when saturated with 95% O2/5% CO2. The muscle was fixed at the ribs in a recording chamber (Figure 1) on an upright microscope, viewed under low magnification (12.5x) via a CCD camera and video monitor. A myograph wire attached to the muscle tendon was connected to a Harvard isometric strain gauge transducer to record muscle tension. The transducer output was amplified and filtered before digitising and computer storage. The y-axis gain was varied in order to show twitch tension from different preparations on a similar scale and so has arbitrary scaling. The phrenic nerve was stimulated using a bipolar stimulating electrode placed at the nerve entry point to the muscle with supra-maximal rectangular voltage pulses of 0.2 ms duration at a frequency of 0.5 Hz. Strain gauge transducer and stimulating electrode were mounted on micromanipulators in order to allow accurate positioning relative to the muscle. Acetylcholine, tubocurarine, and sulfoxaflor were applied by manually switching taps controlling solutions flowing to the inflow manifold of the recording chamber.

Results

Initial Experiments

Initial experiments were used to assess the viability of the preparation, the stability of the twitch response, contracture in response to bath-applied agonist, ACh, and block of muscle twitch and contracture by the nicotinic antagonist tubocurarine. Recorded measures include 1) changes in muscle twitch, and 2) muscle contracture following test material application to the bath perfusion system. Muscle twitch tension reflects phrenic nerve action potential-evoked brief contraction of the diaphragm and is displayed as an upward deflection of the recording trace. Muscle contracture reflects a test material-induced prolonged contraction of the diaphragm associated with increased muscle tension beyond that evoked *via* electrical stimulation of the phrenic nerve. In each experiment, a period of at least 3 minutes of stable baseline and twitch tension (as illustrated in Figure 2A and B) was recorded before application of any drugs. Preparations were viable for between 1 and 3 hours and could respond repeatedly to drug application. Figure 2A shows contractile responses of the new-born rat diaphragm to repeated application of ACh (100 µM) and block of the muscle twitches by tubocurarine (10 µM). Individual muscle twitches, illustrated on a faster time base, can be seen more clearly as upward deflections of the recording trace, in the lower panels of Figure 2 (B and C), while the muscle contracture in response to ACh produces a sustained increase in muscle tension with a concomitant decreased twitch response (A and C). In Figure 2B it can be seen that the muscle twitch in response to phrenic nerve stimulation is stable before test material application and the contracture in response to ACh and block of muscle twitches by the nicotinic antagonist, tubocurarine, are as normally expected.

Figure 4.11.3.1.Study 6.1 (DAR Figure 1.): Diagram of neonatal rat phrenic nerve-hemidiaphragm recording apparatus.

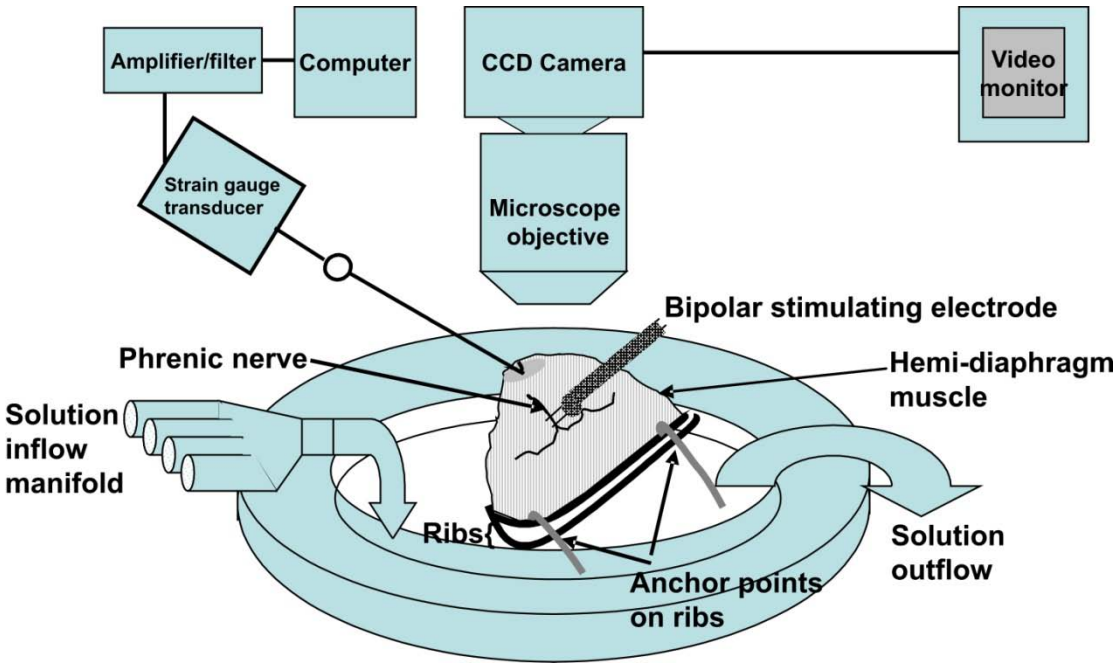
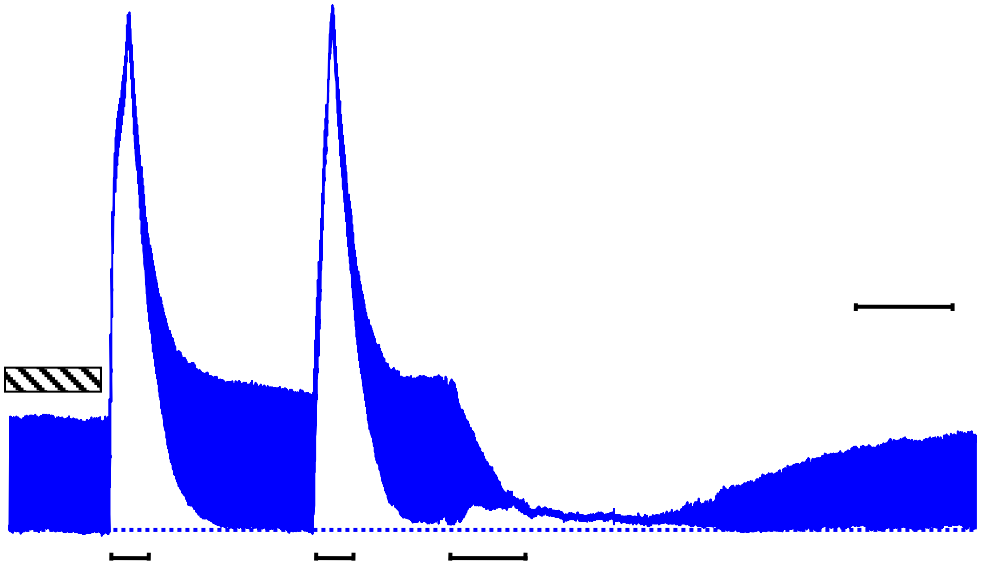


Figure 4.11.3.1.Study 6.2A

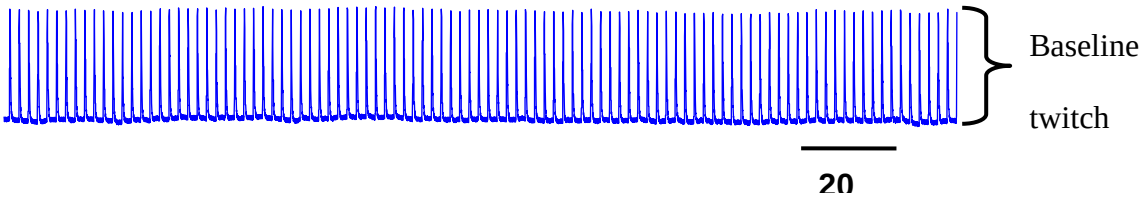
Representative recording of responses of the isolated rat phrenic nerve-hemidiaphragm



preparation to phrenic nerve stimulation and bath application of 100 µM ACh or 10 µM

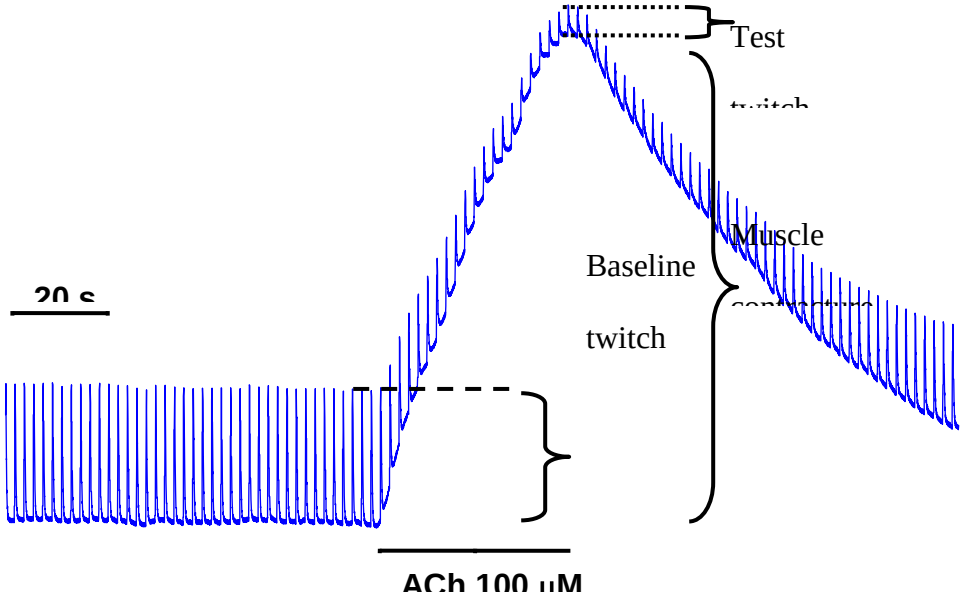
tubocurarine. Increase in muscle tension is recorded as an upward deflection of the trace. Muscle contraction is recorded as a sustained increase in muscle tension when ACh was applied for approximately 100 seconds via the bath perfusion system while the phrenic nerve was stimulated once every 2 seconds to evoke each muscle twitch.

Figure 4.11.3.1.Study 6.2B



The region indicated by the hatched bar in panel A is shown on an expanded time scale in B illustrating that muscle twitches are stable.

Figure 4.11.3.1.Study 6.2C



Expanded time scale display of the second ACh response in panel A shown to clearly illustrate the muscle contracture and decrease in muscle twitch tension induced by ACh. In this example, in the presence of ACh the muscle twitch tension is reduced to 24% of the baseline.

1. Concentration-dependence of the contracture produced by sulfoxaflor

As illustrated in a representative trace in Figure 3, in four separate preparations 1mM sulfoxaflor consistently produced a contracture of the neonatal diaphragm muscle and a decrease in muscle twitch response similar in magnitude to that observed with ACh (100 µM). This *ex-vivo* response was concentration-dependent and qualitatively matched the concentration-dependence

expected from the sulfoxaflor dose-response relationship measured using recombinant rat embryonic ($(\alpha1)_2\beta1\gamma\delta$) nAChRs expressed in *xenopus* oocytes. These data demonstrate that sulfoxaflor can effectively evoke a concentration-dependent contracture of the rat diaphragm from new-born rats.

Figure 3A

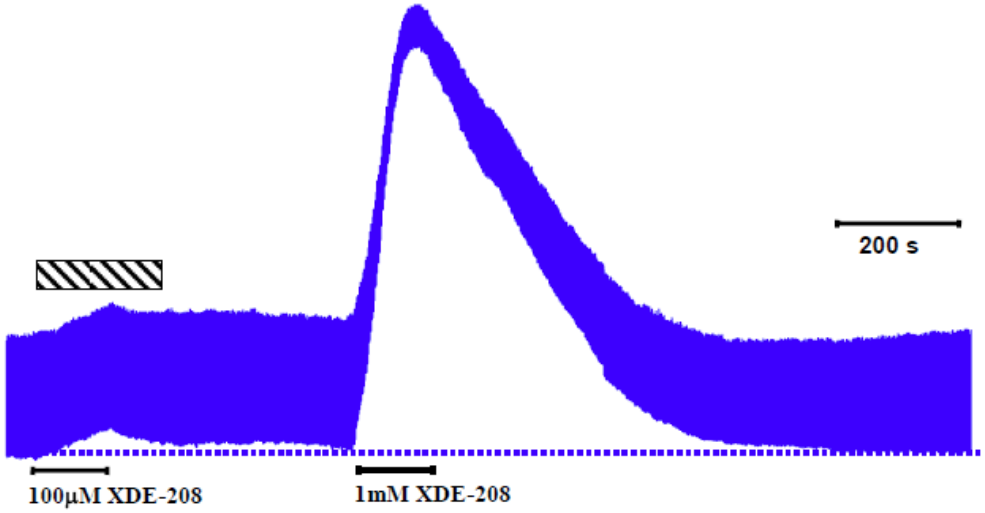
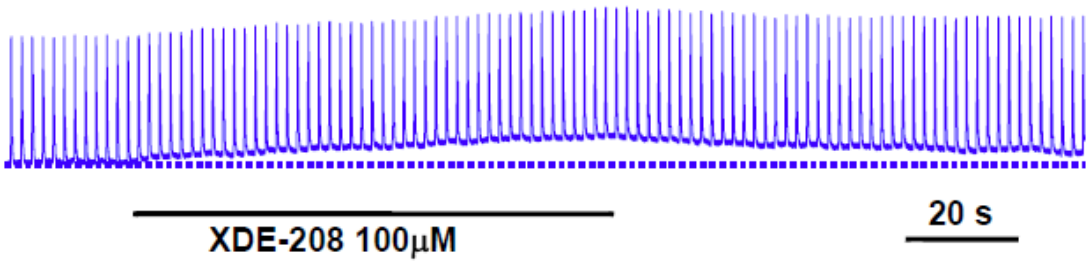


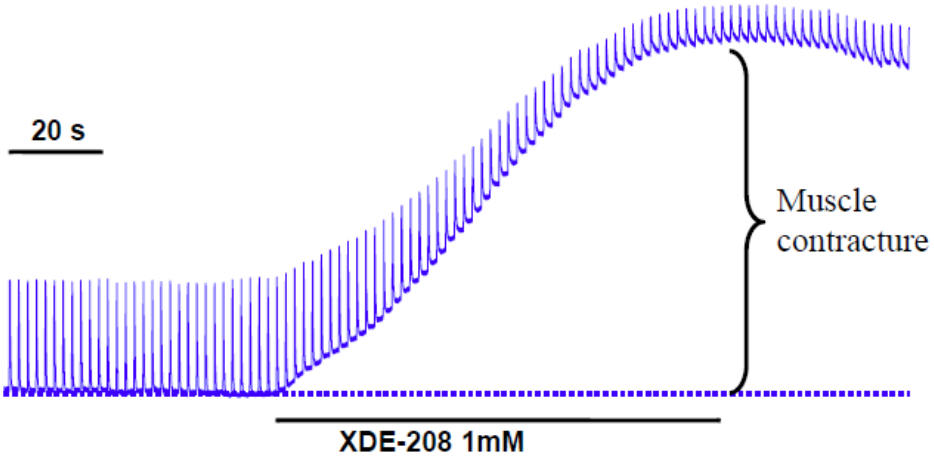
Figure 4.11.3.1.Study 6.3A Concentration-dependence of the muscle contracture in response to sulfoxaflor.

Figure 4.11.3.1.Study 6.3B



On an expanded time scale the region indicated by the hatched box in panel A is reproduced showing individual muscle twitches evident as brief upward deflections of the recording trace (occurring once every 2 seconds). It is clear that at 100 µM sulfoxaflor there was little effect on twitch tension (in five preparations twitch tension was 99 ± 1.5% of control in the presence of 100 µM sulfoxaflor).

Figure 4.11.3.1.Study 6.3C



Expanded time scale display of the second sulfoxaflor response in panel A shown to clearly illustrate the muscle contracture and the accompanying decrease in twitch tension induced by sulfoxaflor. In five preparations the muscle twitch response decreased to 34 ± 3.2% of control during responses to 1 mM sulfoxaflor.

The muscle twitch in response to phrenic nerve stimulation decreased by 66 ± 3.2% during the diaphragm contracture response to a high concentration of sulfoxaflor. Such a contracture-associated decrease in twitch tension could account for the breathing difficulties observed in some neonatal rats after birth, since breathing requires brief nerve-evoked contractions of the diaphragm and there is a well established direct correlation between muscle twitch block and inhibition of mammalian breathing muscles (Paton, 1951; Smith, 1989; Bowman, 1990; Fortier, 2001).

2. Block of the response to sulfoxaflor by tubocurarine

In four separate preparations, the response to sulfoxaflor was shown to be antagonised by the nicotinic antagonist, tubocurarine. Figure 4 illustrates an experiment where following responses to 100 µM and 1 mM sulfoxaflor, 1 mM sulfoxaflor was co-applied with 10 µM tubocurarine.

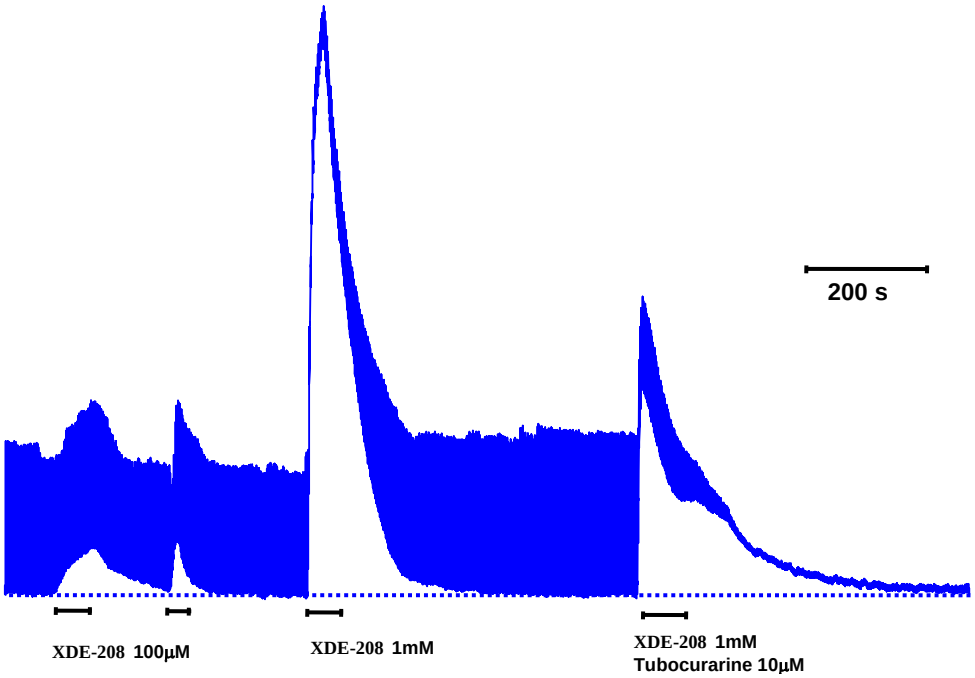


Figure 4.11.3.1.Study 6.4 In this preparation 10 µM tubocurarine was found to block about half of the contracture when co-applied with sulfoxaflor, likely due to rate-limiting diffusion of the antagonist into the tissue.

Although 10 µM tubocurarine might have been expected to block almost 100% of the sulfoxaflor contracture during co-application, it is likely that 1 mM sulfoxaflor will diffuse into the tissue much faster than 10 µM tubocurarine, so sulfoxaflor could activate receptors before they are blocked by tubocurarine (10 µM). This idea was tested by using a pre-incubation protocol (Figure 5).

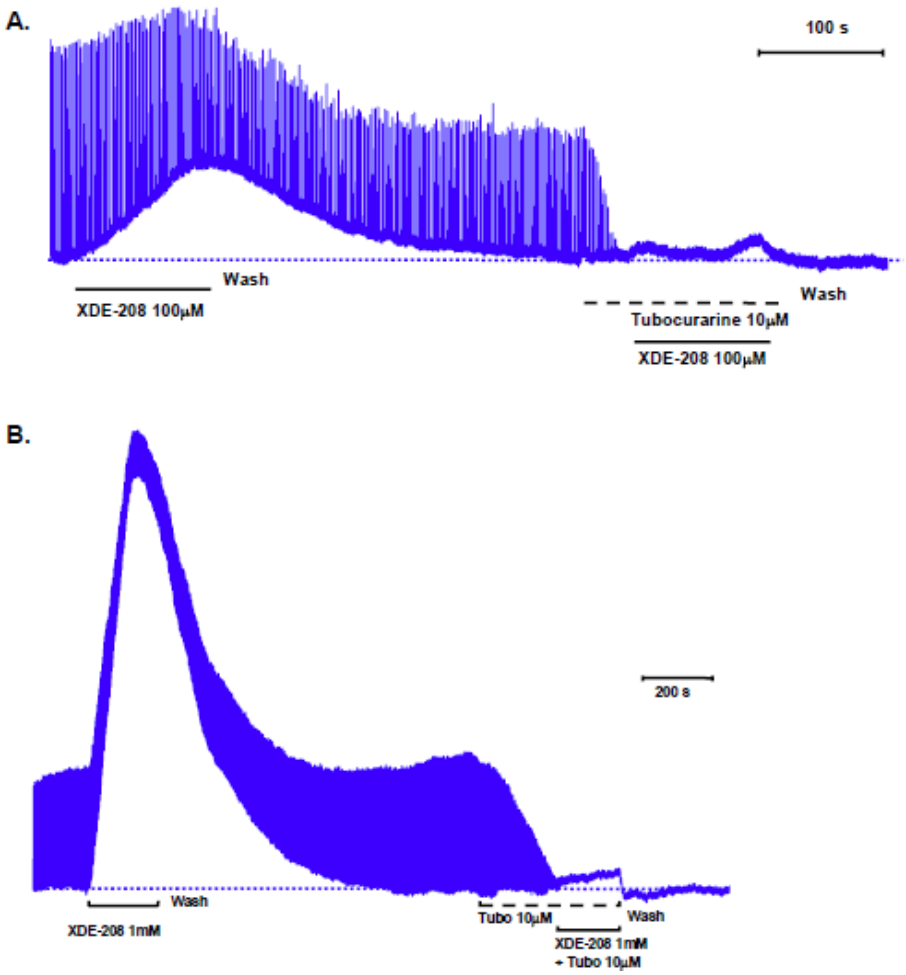


Figure 4.11.3.1.Study 6.5: Pre-application of 10µM tubocurarine (Tubo) effectively blocks the muscle twitches and antagonises responses to **(A)** 100µM or **(B)** 1mM sulfoxaflor.

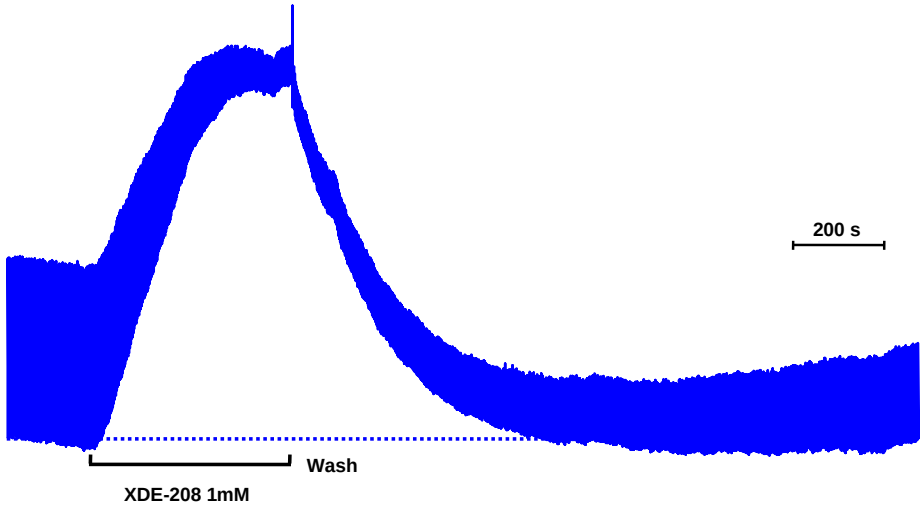
These results demonstrate that contractures caused by sulfoxaflor are blocked by the nicotinic antagonist, tubocurarine (Figures 4 and 5), indicating that sulfoxaflor is acting *via* the nAChR and not *via* a post-receptor mechanism.

4. Response to prolonged sulfoxaflor application

During application of a high concentration of sulfoxaflor (1mM), the muscle contracture is sustained

as illustrated in Figure 6, suggesting little desensitization of the muscle nAChRs in response to this agonist. A lack of desensitization of the foetal muscle-type nAChR during exposure to sulfoxaflor was also shown in the report by Millar (B6.6.12.5).

Figure 4.11.3.1.Study 6.6: Prolonged application (7 minutes) of 1 mM sulfoxaflor shows a



sustained contracture by the diaphragm muscle and a reduction of the muscle twitches by 82% of the baseline.

Although the muscle contracture is sustained in the presence of sulfoxaflor, the muscle recovers normal function on removal of sulfoxaflor from the solution bathing the muscle. The action of sulfoxaflor is, therefore, freely reversible with no evidence of any irreversible effect on muscle function or on neuromuscular transmission. The sustained contracture observed here during prolonged application of sulfoxaflor suggests that this could be the mechanism causing the foetal limb contractions seen *in vivo* while the contracture-associated decrease in phrenic nerve-evoked muscle twitches, which is closely correlated with inhibition of the breathing muscles in experimental animals (Paton, 1951), is the likely mechanism underlying compromised respiration and reduced neonatal survival seen *in vivo*.

Conclusions

The results of these qualitative experiments demonstrate that sulfoxaflor causes a concentration-dependent contracture of the new-born rat diaphragm via activation of muscle-type nAChRs. Prolonged application of sulfoxaflor caused a sustained muscle contracture and contracture-associated decrease in muscle twitch that is considered analogous to the situation in vivo that resulted in poor survival after birth. Thus the results described herein are entirely consistent with and add additional support to the hypothesis that sulfoxaflor causes neonatal death (and foetal abnormalities) via activation of the foetal muscle nAChR.

References

Paton, W. and E. Zaimis (1951) The action of D-tubocurarine and of decamethonium on respiratory and other muscles in the cat. J. Physiol 112:311-331

Smith, C., F. Doneti and D. Bevan (1989) Effects of succinylcholine at the masseter and adductor pollicis muscles in adults. Anesth. Analg. 69:158-162

Bowman, W. (1990) Pharmacology of Neuromuscular Function, 2nd ed. London: Wright

Fortier, P., R. Robitaille, and F. Donati (2001) Increased sensitivity to depolarizing and nondepolarizing neuromuscular blocking agents in young rat hemidiaphragms. Anesthesiology 95:478-484.

Study 7: Foetal Lung contracture

Dietary administration of 1000 ppm sulfoxaflor to Crl:CD(SD) rats during gestation has been previously shown to cause neonatal pup death. In order to determine if morphological alterations (e.g., increased collagen deposition) in any region of the lungs were responsible for pup death, one foetus/sex from five control and four 1000 ppm litters (18 samples total) from the definitive developmental toxicity study were collected and preserved in neutral, phosphate buffered 10% formalin. Sections from these preserved tissues were processed such that each slide contained sections of the trachea, bronchi, bronchioles, and alveoli. Slides were stained with haematoxylin and eosin and evaluated for histopathological changes. Tissues were archived with the developmental toxicity study.

To detect any morphological abnormalities, including increased collagen deposition in the pulmonary tract, of rat foetuses exposed *in utero* to the high-dose of 1000 ppm sulfoxaflor which may have been contributory to treatment-related increase in neonatal pup mortality.

Two formalin fixed foetuses (one male and one female) per dam from the control group and from dams fed 1000 ppm sulfoxaflor were randomly selected. Five control dams and four dams given 1000 ppm were selected, totaling ten control foetuses and eight sulfoxaflor exposed foetuses. The trachea and the lungs of these selected foetuses were routinely processed for histology, sections cut at 5-6 microns thick, stained with haematoxylin and eosin, and examined by a veterinary pathologist.

There were no sulfoxaflor induced lesions in the trachea, bronchi, bronchioles and alveoli in any of the treated foetuses examined. There were no treatment-related increases in collagen deposition around the airways or alveolar walls or any other changes. All observations were considered within normal limits. Therefore, histopathologic examination of the trachea and lungs of selected foetuses from dams given 1000 ppm sulfoxaflor from GD 6-21 did not reveal any morphologic abnormalities in the trachea or within the lungs that could have contributed to 1000 ppm sulfoxaflor induced neonatal mortality in rat pups.

Report:	Histopathological Evaluation Of Fetal Lung Samples From The Developmental Toxicity Study In Crl:Cd(Sd) Rats.
Author:	J. Thomas, Ph.D. and V. A. Marshall, B.S. (2010).
Date of Report:	18 June, 2010
Report Identity:	Study ID: 100124
Testing Facility:	Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan, 48674
GLP	The study is not GLP compliant. However, all experiments were done according to GLP standards.
Test Substance:	XDE-208 (95.6% (wt/wt); as two diastereomers in 50/49.5% ratio.
Batch:	E2162-34

Guidelines: Non-guideline
Deviations: Not applicable
Acceptable: Yes

Dam # 6351	0		
Male foetus #2		NVL	NVL

Introduction

Dietary administration of 1000 ppm sulfoxaflor to Crl:CD(SD) rats during gestation has been previously shown to cause neonatal pup death. In order to determine if morphological alterations (e.g., increased collagen deposition) in any region of the lungs were responsible for pup death, a histological examination of lung tissues from representative samples of control and treated foetal lungs was carried out.

Female foetus #4		NVL	NVL
Dam # 6352	0		
Male foetus # 1		NVL	NVL
Female foetus #3		NVL	Extramedullary hematopoiesis, multifocal, very slight
Dam # 6353	0		
Male foetus # 1		NVL	NVL
Female foetus #5		NVL	Extramedullary hematopoiesis, focal, very slight
Dam # 6354	0		
Male foetus # 3		NVL	NVL
Female foetus #1		NVL	Extramedullary hematopoiesis, multifocal, very slight
Dam # 6428	1000		
Male foetus # 6		NVL	NVL
Female foetus #2		NVL	NVL
Dam # 6429	1000		
Male foetus # 5		NVL	NVL
Female foetus #2		NVL	NVL
Dam # 6430	1000		
Male foetus # 1		NVL	Inflammation, subacute, multifocal, peribronchiolar, very slight
Female foetus #5		NVL	NVL
Dam # 6432	1000		
Male foetus # 5		NVL	Extramedullary hematopoiesis, focal, very slight
Female foetus #1		NVL	NVL

Table 4.11.3.1.Study 7.1:

Materials and Methods

One foetus/sex from five control and four 1000 ppm litters (18 samples total) from the definitive developmental toxicity study were collected and preserved in neutral, phosphate buffered 10% formalin. Sections from these preserved tissues were processed such that each slide contained sections of the trachea, bronchi, bronchioles, and alveoli. Slides were stained with hematoxylin and eosin and evaluated for histopathological changes.

Results

Histopathologic observations of fetal trachea and lungs from ten control foetuses and eight sulfoxaflor exposed foetuses are presented in Table B.6.6.12.7-1.

There were no sulfoxaflor induced lesions in the trachea, bronchi, bronchioles and alveoli in any of the treated foetuses examined. There were no treatment-related increases in collagen deposition around the airways or alveolar walls or any other changes. All observations were

considered within normal limits.

Conclusion

Histopathologic examination of the trachea and lungs of selected foetuses from dams given 1000 ppm sulfoxaflor from GD 6-21 did not reveal any morphologic abnormalities in the trachea or within the lungs that could have contributed to 1000 ppm sulfoxaflor induced neonatal mortality in rat pups

Study 8: Human Relevance Framework

The following section summarises the notifiers evaluation of the reproductive and developmental data including the MoA studies according to the Bradford-Hill criteria and the subsequent application of the Human Relevence Framework.

Sulfoxaflor, an insecticide that operates *via* the insect nicotinic acetylcholine receptor (nAChR), causes foetal abnormalities (primarily limb contractures) and death in neonatal rats, but not rabbits, following high dose dietary exposure during gestation in regulatory guideline studies. It has been proposed that these effects have a single mode of action (MoA) mediated *via* the rat foetal-type muscle nAChR through the following key events: (1) binding to the receptor, (2) agonism (activation) at the receptor, causing (3) sustained muscle contracture in the near-term foetus and neonatal offspring. This sustained muscle contracture results in limb contractures, bent clavicles, and reduced function of the diaphragm, which compromises respiration in offspring at birth and reduces neonatal survival. The three key events have been evaluated in a series of MoA studies aimed at examining the causality of sulfoxaflor's induction of these effects as observed in the regulatory guideline studies. The document represents the weight of evidence approach used to evaluate the data based upon the Bradford-Hill criteria followed by subsequent application in a Human Relevance Framework (HRF). The conclusion from this evaluation is that there is a high level of confidence that the observed sulfoxaflor-induced foetal abnormalities and neonatal offspring death in rats occur *via* a single MoA comprised of sustained activation of the rat foetal-type muscle nAChR resulting in muscle contracture. In addition, this MoA is not considered relevant to humans based upon available data demonstrating fundamental qualitative differences in sulfoxaflor agonism at the rat *versus* the human muscle nAChR where agonism occurs at the rat foetal-type, but not the human foetal or adult-type, muscle nAChR.

Report:	Sulfoxaflor: Mode of action evaluation and human relevance framework analysis for Sulfoxaflor-induced foetal abnormalities and neonatal death in rats.
Author:	R. G. Ellis-Hutchings, Ph.D., R. J. Rasoulpour, Ph.D., C. Terry, Ph.D., B.

B. Gollapudi, Ph.D., and R. Billington, M.Sc., DABT, DRCPath
Date of Report: 7th December 2010
Report Identity: Study ID: 100290

Testing Facility: Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan, 48674
Acceptable: Yes

Summary

Sulfoxaflor, an insecticide that operates *via* the insect nicotinic acetylcholine receptor (nAChR), causes foetal abnormalities (primarily limb contractures) and death in neonatal rats, but not rabbits, following high dose dietary exposure during gestation in regulatory guideline studies. It is proposed that these effects have a single mode of action (MoA) mediated *via* the rat foetal-type muscle nAChR through the following key events: (1) binding to the receptor, (2) agonism (activation) at the receptor, causing (3) sustained muscle contracture in the near-term foetus and neonatal offspring. This sustained muscle contracture results in limb contractures, bent clavicles, and reduced function of the diaphragm, which compromises respiration in offspring at birth and reduces neonatal survival. The three key events have been evaluated in a series of MoA studies aimed at examining the causality of sulfoxaflor's induction of these effects as observed in the regulatory guideline studies. This document represents the weight of evidence approach used to evaluate the data based upon the Bradford-Hill criteria followed by subsequent application in a Human Relevance Framework (HRF). The conclusion from this evaluation is that there is a high level of confidence that the observed sulfoxaflor-induced foetal abnormalities and neonatal offspring death in rats occur *via* a single MoA comprised of sustained activation of the rat foetal-type muscle nAChR resulting in muscle contracture. In addition, this MoA is not relevant to humans based upon available data demonstrating fundamental qualitative differences in sulfoxaflor agonism at the rat *versus* the human muscle nAChR where agonism occurs at the rat foetal-type, but not the human foetal or adult-type, muscle nAChR.

Introduction

Sulfoxaflor, [1-(6-Trifluormethylpyridin-3-yl)ethyl)](methyl)-oxido-λ^4-sulfanylidenecyanamide) is a compound with insecticidal properties mediated *via* its agonism at the highly abundant insect nicotinic acetylcholine receptor (nAChR) (Zhu et al., 2010). During the conduct of regulatory guideline mammalian toxicology studies, developmental effects were observed in rats following dietary sulfoxaflor exposure, but not rabbits, including offspring death and near-term foetal abnormalities (forelimb flexure, hindlimb rotation, and bent (misshapen) clavicle bones (Figure 1). More specifically, the rat limb effects were without changes in the associated skeletal bone structure while the offspring death occurred prior to postnatal day (PND) 4. Henceforth, these effects will be summarily classified as occurring in neonatal offspring. A subsequent cross-fostering study demonstrated that the neonatal offspring death was due to gestational, and not lactational, exposure. Additional non-guideline investigative toxicity studies demonstrated that the rat limb and survival effects were inducible with one or two days of exposure before birth, with the limb effects being rapidly reversible upon withdrawal of sulfoxaflor exposure in surviving pups.

An additional finding of convoluted/hydroureters, with no associated dilatation of the renal pelvis, was seen in the rat developmental toxicity study (Rasoulpour et al., 2010d). The ureter effects were consistent with transient urinary bladder contraction preventing urine outflow from

the ureters leading to their distention and convolution. This low incidence finding is regarded in the literature as a minor variant and is readily reversible after birth (Solecki et al., 2003), a fact that is supported by two critical window studies (Rasoulpour and Zablotny, 2010; Rasoulpour and Zablotny, 2010 a, b). The ureter effects were considered of minor concern, had no sequelae in the sulfoxaflor studies, and therefore will not be discussed further.

The aggregation of the relevant toxicity and mode of action studies of sulfoxaflor here within provides the context for which to evaluate the proposed mode of action (MoA) and detailed MoA analysis. This analysis is based on the hypothesis that following exposure, sulfoxaflor demonstrates sustained agonism to the rat foetal-type muscle nAChR causing sustained muscle contracture. Contracture is defined as an abnormal contraction of muscle tissue rendering it highly resistant to passive stretching, ultimately resulting in the limb contractures and diaphragm-related death observed in neonatal offspring. Also incorporated into this analysis is a consideration of alternative modes of action which also result in limb abnormalities and/or death in neonatal offspring. This is then followed by a human relevance framework analysis addressing the relevance of the rat developmental effects to humans.

A. HYPOTHESIS FOR SULFOXAFLOR-INDUCED MUSCLE CONTRACTURE AND DEATH IN NEONATAL OFFSPRING

As discussed in the previous section, dietary exposure to Sulfoxaflor during gestation in rats results in treatment-related limb contracture and death in neonatal offspring. The guideline studies demonstrating these effects include a rat prenatal developmental toxicity study (Rasoulpour and Marshall, 2010) in which external abnormalities of forelimb flexure and hindlimb rotation (contracture effects without changes in the associated skeletal bone structure) and bent (misshapen) clavicle bone were observed in foetuses, and rat reproductive toxicity studies (Rasoulpour et al., 2010b; Rasoulpour et al., 2010e) where neonatal offspring death was observed (on or before postnatal day (PND) 4). Non-guideline follow-up studies demonstrated limb contracture in neonatal offspring inducible with as little as one or two days of exposure before birth, and were rapidly reversible upon withdrawal of treatment (Rasoulpour and Zablotny, 2010a; Rasoulpour and Zablotny, 2010b). In addition, a cross-fostering study demonstrated that the neonatal offspring death was due to gestational, and not lactational, exposure (Rasoulpour and Zablotny, 2010c). While these developmental effects were observed in the rat, similar effects were not observed in a rabbit developmental toxicity or perinatal survival study (Kuhl, 2010; Rasoulpour et al., 2010c). Similar maternal and foetal blood data showed that the interspecies difference between rats and rabbits was not due to toxicokinetics; therefore, the species difference must be due to toxicodynamics.

Given that the rat limb skeletal structures were normal in foetuses with limb contracture abnormalities it was considered likely that the observed limb contracture and bent clavicles (Figure 1) resulted from sulfoxaflor's action on the neonatal offspring muscle. Supporting this hypothesis is the observation that foetuses in the developmental toxicity study exhibited a hunched posture, consistent with generalised muscle contracture, and the fact that the clavicle (collar) bone is dependent upon normal shoulder muscle function for proper development during the late foetal period (Pai, 1965; Tran and Hall, 1989). Similar to the limb and the shoulder muscles, action of sulfoxaflor on the main muscle involved in breathing, the diaphragm (Vander et al., 2001), could result in abnormal neonatal respiration and mediate the neonatal deaths.

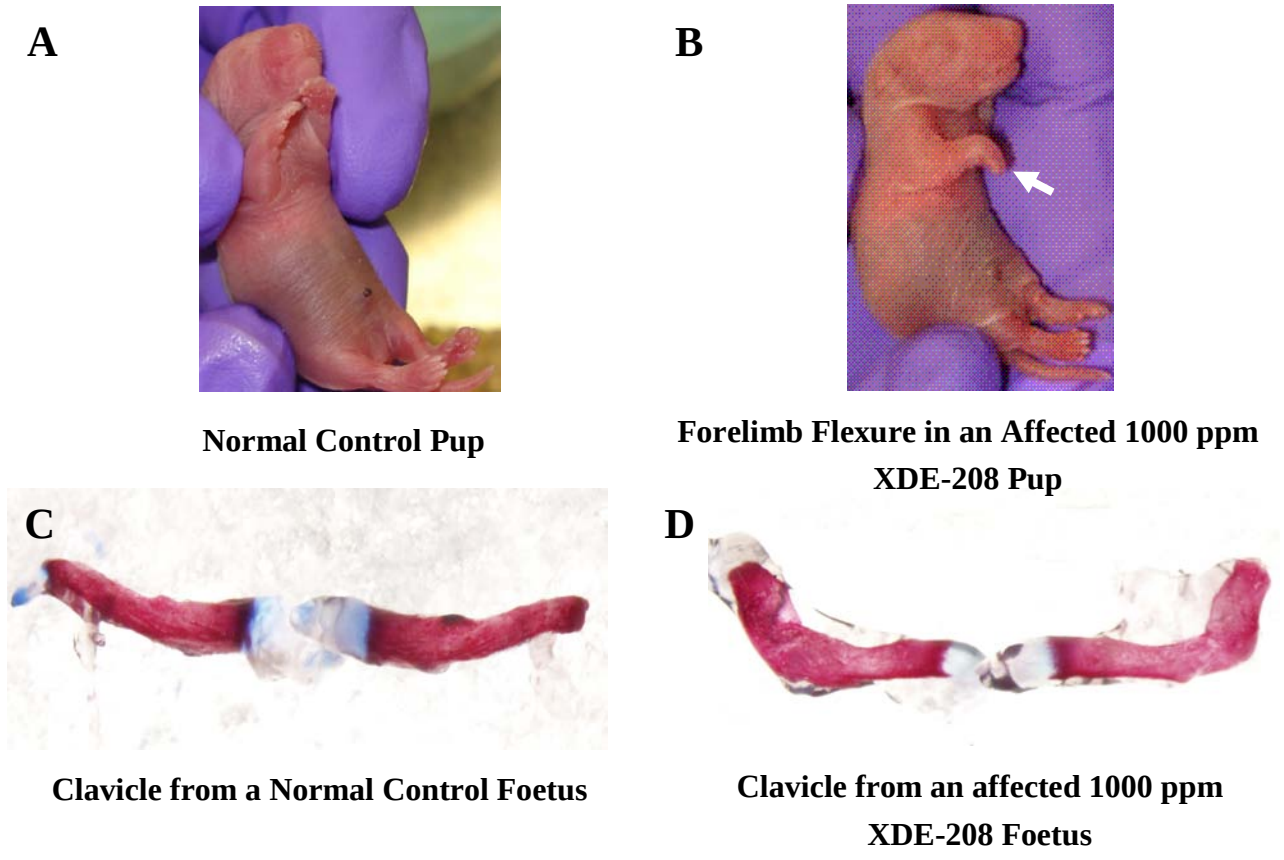


Figure 4.11.3.1.Study 8.1 (DAR Figure B.6.6.12.9-1.): Photomicrographs of representative limb contracture (e.g. forelimb flexure) (B) and bent clavicles (D) in neonatal offspring of rat dams exposed to 1000 ppm sulfoxaflor relative to controls (A and C) (Rasoulpour et al., 2010b; Rasoulpour and Zablotny, 2010a).

1. Rat Muscle Contracture and associated death in neonatal offspring occur via the same mode of action.

A single MoA is considered to be responsible for the neonatal offspring findings as the sulfoxaflor-induced limb contracture and clavicle abnormalities and the neonatal death occurred at the same dose (1000 ppm) with similar incidences. In order for a single mode of action to be plausible, the findings should have a similar incidence across doses from all studies. Figure 2 demonstrates that, based upon similar dose-response curves (non-linear regressions) for both effects, the data for these findings from multiple independent studies have a high degree of correlation (R^2 values of 0.93 and 0.91) thus supporting a single MoA with different apical end points rather than a different MoA for each of the two major effects in rats. The hypothesised single MoA, along with additional supporting evidence, will be discussed in the following sections.

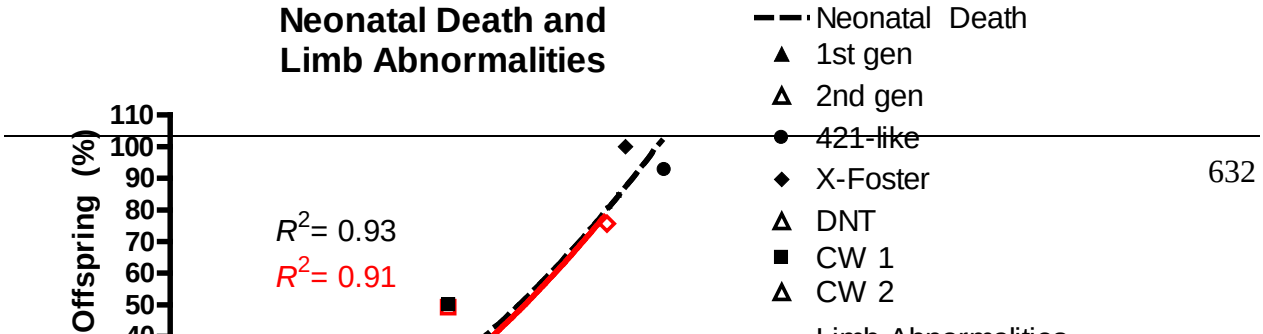


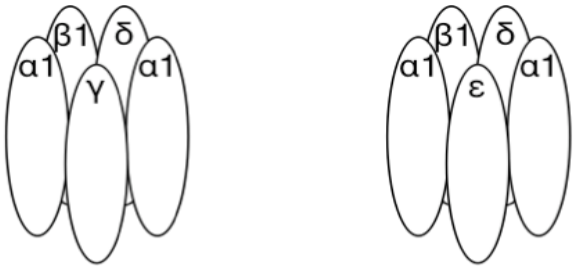
Figure 4.11.3.1.Study 8.2 (DAR Figure B.6.6.12.9-2): Dose-response curves with non-linear regression analyses for the percent of neonatal offspring affected by death and/or limb contracture in rats.

Study type abbreviations: 1st gen and 2nd gen, 1st and 2nd generations of the two-generation reproductive toxicity study; 421-like, reproductive toxicity screening study; X-foster, cross-foster study; CW 1, critical window study phase 1; CW 2, critical window study phase 2; DNT, developmental neurotoxicity study; DT, prenatal developmental toxicity study.

2. Muscle nAChR is hypothesised to mediate sulfoxaflor's developmental effects

As the sulfoxaflor's insecticidal MoA is agonism at the insect nAChR, it was logical to investigate the mammalian muscle nAChR as a biologically plausible target responsible for these effects in the rat. Interestingly, sulfoxaflor's primary environmental metabolite (X11719474), was known not to bind to the insect nAChR (Watson and Young, 2010) and, when tested in developmental or reproductive toxicity studies, did not produce limb contracture abnormalities or neonatal offspring death at dose levels 5-10 times higher than the sulfoxaflor effect levels (Rasoulpour and Marshall, 2010; Rasoulpour et al., 2010a). In both invertebrate and vertebrate species, nAChRs are important neurotransmitter receptors (Sattelle, 1980; Millar and Gotti, 2009) and comprise a diverse family of oligomeric cell-surface receptors assembled from five (of many) subunits that co-assemble in a doughnut-shaped arrangement (Millar and Denholm, 2007; Millar and Gotti, 2009) (Figure 3). In the center of the pentameric arrangement of subunits is a cation-selective ion channel, whereby binding of the endogenous neurotransmitter, acetylcholine, or other agonists stabilizes the open conformation allowing the influx of cations into the cell (agonism). In mammalian muscle cells, nAChRs are expressed at the neuromuscular junction (NMJ) and are composed of five nAChR subunits (α1, β1, γ, δ and ε). Transcription of the γ and ε subunits is differentially regulated during development, with the γ subunit expressed in "foetal" muscle and the ε subunit expressed in "adult" muscle (Mishina et al., 1986) (Figure 3). Muscle nAChRs contain two agonist binding sites, one at the interface of the α1 and δ subunits and another at the interface of the α1 and γ (or ε) subunits (Arias, 2000).

In rats, the muscle nAChR develops functional subunit expression at the NMJ between GD 15 and 17 (Kues et al., 1995) resulting in synchronized foetal limb movements (Robinson and Smotherman, 1988) and diaphragmatic responsiveness between GD 16 and 17 (Bennett and Pettigrew, 1974), the latter being critical for the transition to extrauterine respiration. Replacement of the γ subunit by the ε subunit initiates late during the first postnatal week in rats and is largely complete by the end of the second postnatal week in limb and respiratory muscles (Missias et al., 1996).



Foetal-type nAChR **Adult-type nAChR**

Table 4.11.3.1.Study 8.3 (DAR Figure B.6.6.12.9-3.): Subunit composition of the foetal-type and adult-type muscle nAChR

B. POSTULATED MoA AND KEY EVENTS ASSESSMENT FOR

XDE-208-DEVELOPMENTAL EFFECTS

Given that sulfoxaflor targets the insect nAChR, and that functional expression of the foetal-type NMJ nAChR occurs in late gestation and is involved with limb muscle function, it was hypothesised that the neonatal offspring limb/clavicle abnormalities and death occur *via* a single MoA: Sulfoxaflor's sustained agonism at the foetal-type muscle nAChR and subsequent sustained muscle contracture of the limb, shoulder girdle and diaphragm, respectively. Based upon this hypothesis, a series of MoA studies were designed and conducted accordingly. Study summaries are provided in the Appendix but will be expounded upon in the context of the weight of evidence evaluation for this postulated MoA. This MoA is proposed to progress through the following key events: (1) sulfoxaflor binding and (2) agonism at the foetal-type muscle nAChR, resulting in (3) sustained agonism/sustained muscle contracture in neonatal offspring (foetus and pup) (Table 1). This sustained muscle contracture results in limb contracture, bent clavicles, and abnormal neonatal respiration after birth resulting in neonatal offspring death (Figure 4). Alternative MoAs were ruled out based upon a thorough review of the literature and their consideration is discussed following the postulated MOA assessment.

Table 4.11.3.1.Study 8.1 (DAR Table B.6.6.12.9-1.): Key Events for the Postulated Mode of Action of Sulfoxaflor-induced Muscle Contracture Abnormalities and Associated Death in Neonatal Offspring

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(1) Binding to the foetal-type muscle nAChR
(2) Agonism at the foetal-type muscle nAChR
(3) Sustained agonism/sustained muscle contracture

Diaphragm (abnormal neonatal aspiration resulting in death)

Limb (forelimb flexure and hindlimb rotation) and shoulder girdle (bent clavicles)

Reversal upon discontinuance of treatment

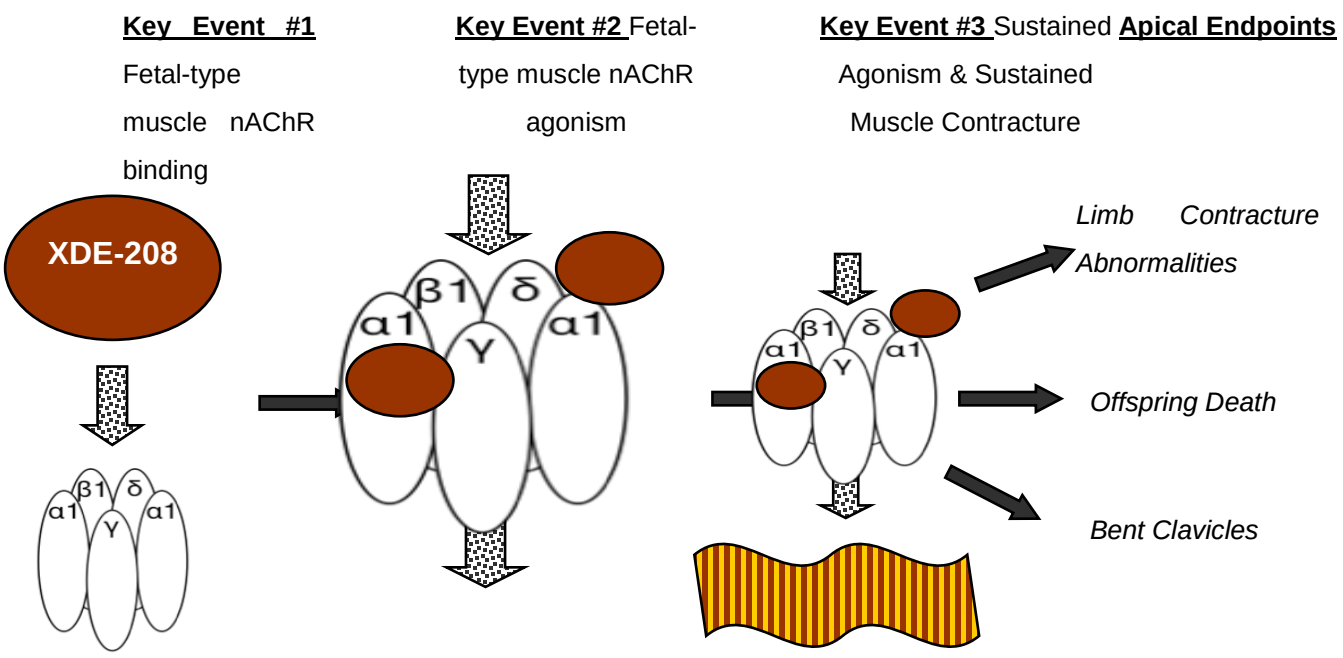


Figure 4.11.3.1.Study 8.4 (DAR Figure B.6.6.12.9-4.): Key Events for the Postulated Mode of Action of sulfoxaflor-induced Muscle Contracture Abnormalities and Associated Death in Neonatal Offspring

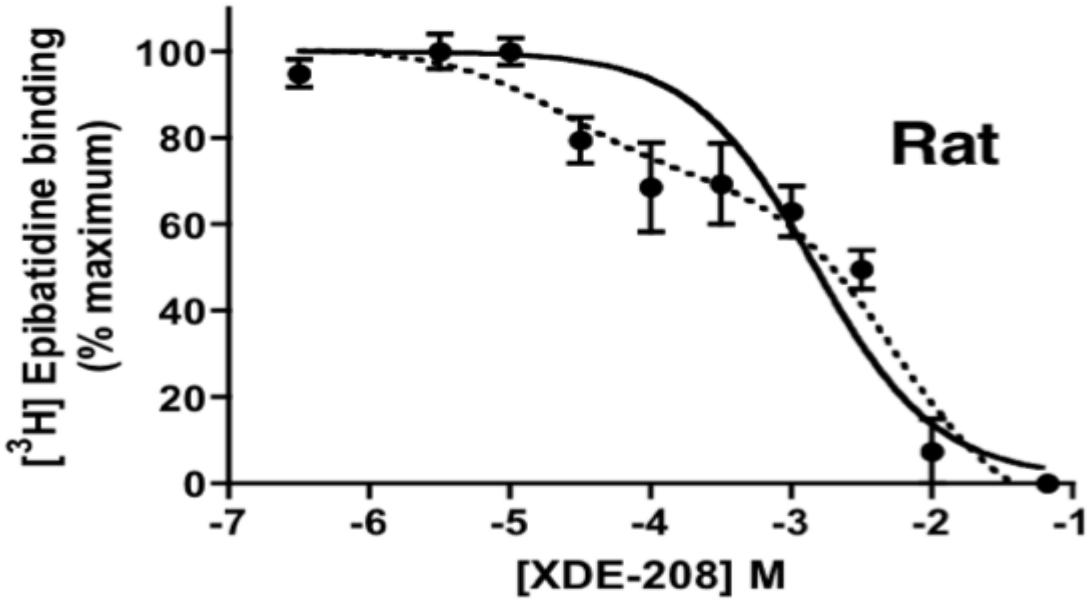
These three key events have been evaluated in a series of MoA studies aimed at examining the causality of sulfoxaflor's induction of these effects as observed in the regulatory guideline studies. The remainder of this document represents the weight of evidence approach used to evaluate the data based upon the Bradford-Hill criteria (Hill, 1965) followed by subsequent application in a Human Relevance Framework (HRF). This approach has been utilised in the analysis of carcinogen MoAs and their human relevance. More recently effort has been directed towards extending this approach to non-cancer MoA analyses (Corley et al., 2005; Foster, 2005; Holson et al., 2005; Kavlock and Cummings, 2005) with several addressing human relevance. Standardization of the approach has also been recommended by the International Programme on Chemical Safety (IPCS) (Boobis et al., 2008). To date there have been no published MoAs and HRF assessments for foetal-type muscle contracture abnormalities and associated neonatal offspring death.

Dose response and reversibility data for the postulated key events and the associated end points of forelimb flexure, hindlimb rotation, bent clavicle and neonatal death are described within each key event analysis as well as summarised in Tables 8 and 9. The MoA analysis is composed of data from a variety of different study designs in different settings, including *in vivo, ex vivo* and *in vitro* studies, thereby providing a robust demonstration of the consistency of sulfoxaflor's MoA in the rat. Nine independent studies are included in the assessment with many including dose-response data allowing for strong evidence of causality through the demonstration of clear dose-response relationships with each of the key events. Toxicokinetic analysis was incorporated in the majority of the *in vivo* toxicology studies in order to provide a measure of internal dose, which allows direct comparison between the *in vivo* data to the generated *in vitro* and *ex vivo* MoA data. In order to evaluate specificity and strength of the association of the postulated MoA, reversibility tests and extensive evaluations of an agent inactive at the insect

nAChR have been included, respectively.

Key Event No. 1: Specific binding to the foetal-type muscle nAChR

In mammals, nAChRs in muscle tissue are expressed post-synaptically, where they are responsible for contraction of muscle in response to the release of acetylcholine from the pre-synaptic nerve terminal. To assess sulfoxaflor's ability to bind to the foetal-type muscle nAChR, as well as portions of key event No. 2 (agonism to the foetal-type muscle nAChR), a collaboration with Professor Neil S. Millar, Ph.D., the head of molecular pharmacology at University College London, UK (Millar, 2010) was established. Forelimb muscle tissue was isolated from GD 21 rats, homogenised, and differentially centrifuged to isolate the cell membrane fraction containing the foetal-type muscle neuromuscular junction nAChRs. Competitive radioligand binding experiments were conducted using these preparations with a range of sulfoxaflor concentrations. The data obtained in this study provided clear and direct evidence that sulfoxaflor binds to foetal-type muscle nAChRs (Figure B.6.6.12.9-5, Table B.6.6.12.9-2). Thresholded, dose-dependent binding was demonstrated using this approach with the lowest tested concentration having no apparent binding while incubation of the receptor pools with higher concentrations of sulfoxaflor completely displaced the high affinity foetal-type muscle nAChR binding of [^{3}H]-epibatidine, thereby showing specificity of sulfoxaflor to nAChR binding site(s).



ıflor: The
ıples were
incubated with [^{3}H]-epibatidine (30 nM) in the presence of a range of concentrations of sulfoxaflor. Data are means ± SEM of 3-4 independent experiments, each performed with triplicate samples. Levels of radioligand binding are

normalised to the level of specific binding observed in the absence of sulfoxaflor. Concentrations are plotted as log molar concentrations (Millar, 2010).

Concentrations of sulfoxaflor causing half-maximal displacement (IC_{50} concentrations) of 30 nM [^{3}H]-epibatidine were determined. By fitting the data to a single binding site model (i.e. assuming the ligand would have similar affinity for both nAChR agonist binding sites; one being located at the α-γ interface and the other at the α-δ subunit interface) (solid lines in Figure B.6.6.12.9-5), the IC_{50} estimate for sulfoxaflor is 2.3 mM for rat. The foetal rat muscle binding data were not well fitted using this single binding-site model, therefore the data were fitted with a two-site model (dotted line in Figure B.6.6.12.-5) that revealed different affinities for the two binding sites (0.01 mM and 8.9 mM). The better fit of the rat foetal muscle nAChR experimental data to a two-site model would suggest that sulfoxaflor displaces [^{3}H]-epibatidine from the two nAChR agonist-binding sites (located at the α-γ and α-δ subunit interfaces) with different affinities.

Indirect evidence for binding to the foetal-type muscle nAChR includes demonstrations of 1) functional agonism in Xenopus oocytes expressing recombinant foetal-type muscle nAChRs *in vitro* and 2) *ex vivo* muscle contracture in experiments using CD rat PND 0 phrenic nerve-hemidiaphragm muscle preparations. These collaborative efforts with Drs. Neil Millar (Millar, 2010) and Alasdair Gibb (Gibb, 2010) of the University College London, UK will be discussed in more detail in relation to key events No. 2 (agonism at the foetal-type muscle nAChR) and No.3 (sustained agonism at the foetal-type muscle nAChR and sustained muscle contracture), respectively, but these experiments showed that sulfoxaflor caused concentration-dependent agonism at the foetal-type muscle nAChR and contracture of the newborn rat diaphragm, responses which could only have occurred *via* binding and/or subsequent sustained agonism of sulfoxaflor at this receptor. When the direct and indirect evidence of sulfoxaflor binding to the foetal nAChR are taken together, there is support for nAchR binding being a key event operant in the MoA of sulfoxaflor-induced muscle contracture and associated death in neonatal offspring.

Table 4.11.3.1.Study 8.2 (DAR Table B.6.6.12.9-2): Dose Response for MoA Key Event No. 1: Foetal-type Muscle nAChR Binding

Dose ↓

Media Conc. (µM)	Applied Dose (ppm)	Key Event 1 Foetal-type Muscle nAChR Binding[#]
Study Type		*In vitro*[1]
0.3 µM		5[+]
3 µM		0
10 µM		0
30 µM		21

100 µM	31
100 µM *ex vivo*[2]	(+)
300 µM	31
1000 µM	37
3000 µM	51
10000 µM	100

Percent inhibition of maximum epibatidine binding
1 Foetal muscle tissue homogenates
+ Within background of system and not considered biologically significant
[2] Neonatal diaphragm *ex vivo* electrophysiology
( ) Key event not measured but required for foetal muscle-type-dependent diaphragm contraction

Key Event No. 2: Agonism at the foetal-type muscle nAChR

The radioligand binding experiments described above demonstrate specific binding of sulfoxaflor to foetal-type muscle nAChRs but do not indicate whether the binding of sulfoxaflor results in functional activation of the receptor (i.e., it does not indicate whether sulfoxaflor acts as an agonist on mammalian muscle nAChRs). To examine this question, rat foetal and adult muscle nAChRs were expressed as recombinant receptors by microinjection of cRNA in *Xenopus* oocytes through collaboration with the lab of Professor Neil S. Millar, Ph.D. For these experiments, recombinant foetal-type rat nAChR subunits (5 subunits: with foetal subtype α(2),β,δ,γ or adult subtype α(2),β,δ,ε were expressed in *Xenopus* oocytes that form functional nAChRs. The nAChRs expressed in *Xenopus* oocytes are derived from cloned nAChR subunit cDNAs or cRNAs (rather than from mRNA in muscle cells) and form fully functional pentameric nAChRs expressed on the cell surface. Electrophysiological data obtained from such receptors have been demonstrated to be similar to data obtained from native nAChRs expressed in muscle tissue (Mishina et al., 1986). Due to its correlation with *in vivo* functionality the method has been used in hundreds of research publications to examine the functional properties of ion channels such as the nAChR (Dascal, 1987). It has also been previously demonstrated to confirm agonism of nAChR ligands (Cooper et al., 1996), some of which have been demonstrated to cause limb contracture abnormalities (Forsyth et al., 1996). In the current studies, functional diaphragm responses (membrane currents) were confirmed *via* application of the endogenous agonist acetylcholine (ACh) (Figure B.6.6.12.9-6).

Clear agonist-evoked responses were observed with sulfoxaflor at the rat foetal-type nAChR (Figure B.6.6.12.9-6, Table B.6.6.12.9-3). Consistent with the dose-response data from the sulfoxaflor binding experiments, thresholded, dose-dependent agonism at the foetal-type muscle nAChR was demonstrated; the lowest tested concentration having no agonism while incubation of the oocytes with higher concentrations of sulfoxaflor showed increasing agonism (Figure B.6.6.12.9-7).

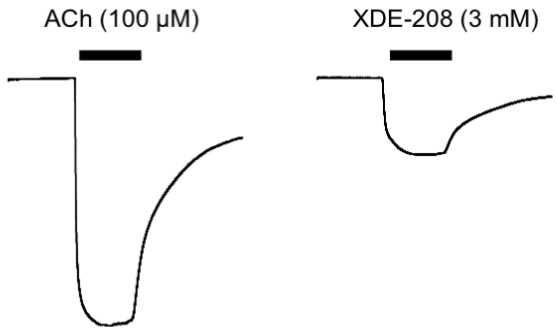


Figure 4.11.3.1.Study 8.6 (DAR Figure B.6.6.12.9-6.): Representative whole-cell current responses in a *Xenopus* oocyte cell expressing rat $(\alpha 1)_2\beta 1\gamma\delta$ nAChRs. Inward currents are show from the same oocyte in response to application of acetylcholine (100 µM) and sulfoxaflor (3 mM). The length of agonist application (5 secs.) is indicated by the horizontal bar. Agonist activation is associated with downward deflection in the trace. Note, recovery of response after ACh or sulfoxaflor were washed off (Millar, 2010).

In these experiments, agonism was sustained and did not diminish with continued exposure indicating that there was no muscle nAChR desensitisation in response to continued agonism. Data from the agonism experiments showed high concordance with the binding experiments thereby providing a demonstration of consistency across experimental designs at the same test concentrations. Evidence for the specificity of sulfoxaflor's agonism to the foetal-type muscle nAChR comes from experiments which showed that despite normal ACh agonist responses, no agonism occurred up to the limit solubility for sulfoxaflor at the rat adult-type muscle nAChR (Figure B.6.6.12.9-7B, Table B.6.6.12.9-3). Furthermore, a sulfoxaflor soil metabolite, X11719474, which is known to be inactive at the insect nAChR (Watson and Young, 2010), did not induce agonism at the foetal-type muscle nAChR (Figure B.6.6.12.9-7A). Consistent with this lack of *in vitro* agonism, X11719474 was previously demonstrated to produce no neonatal pup loss or developmental effects at dose levels 5-10 times higher than the sulfoxaflor effect levels (Table B.6.6.12.9-3)(Rasoulpour et al., 2010a; Rasoulpour and Marshall, 2010). In addition, imidacloprid, an neonicotinoid insecticide which has not been demonstrated to cause contracture-related limb abnormalities, induced very little agonism at the foetal-type muscle nAChR.

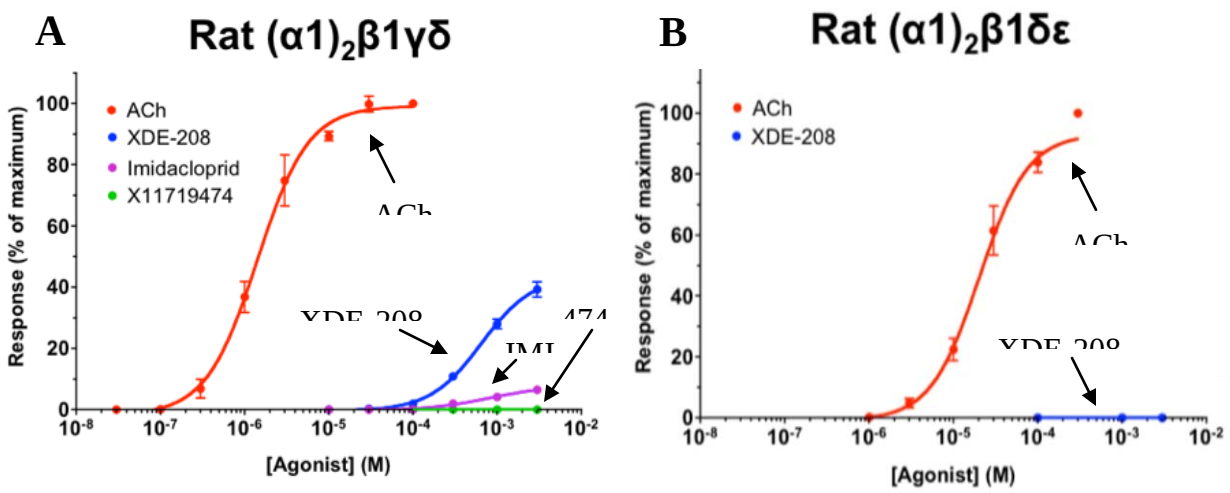


Figure 4.11.3.1.Study 8.7 (DAR Figure B.6.6.12.9-7.): Agonist activation of nAChRs expressed in *Xenopus* oocytes. Data are shown for the rat foetal $(\alpha1)_2\beta1\gamma\delta$ nAChR (A) and rat adult $(\alpha1)_2\beta1\delta\varepsilon$ nAChR (B). AChRs were expressed by microinjection of cRNA in *Xenopus* oocytes. Dose-response curves are shown in which agonist-evoked responses are normalized to the maximal response detected with the endogenous agonist, acetylcholine (ACh). Note that no receptor desensitization occurs, which would have been seen as a diminished agonist response at higher doses. Data points are means of 3-7 responses. Adapted from Millar, 2010

Table 4.11.3.1.Study 8.7 (DAR Table B.6.6.12.9-3.): Dose Response for MoA Key Event No. 2: Foetal-type Muscle nAChR Agonism

Dose

Media Conc (µM)	Applied Dose (ppm)	Key Event 2 Foetal-type Muscle nAChR Agonism[$]
Study Type		*In vitro*[1]
10 µM		0
30 µM		1.0
100 µM		5.1
100 µM *ex vivo*[2]		(+)
300 µM		28
1000 µM		71
3000 µM		100
10000 µM		ND
X11719474 (a sulfoxaflo soil metabolite inactive at the insect nAChR)		
100 to 3000 µM		0
'sulfoxaflor Agonism at the Adult-type Muscle nAChR[3]		
100 to 3000 µM		0

[$]Percent of maximum sulfoxaflor response
[1]*Xenopus* Oocytes-Recombinant Foetal-type Muscle nAChR
[2]Neonatal Diaphragm *Ex Vivo* Electrophysiology
( ) Key event not measured but required for foetal muscle-type-dependent diaphragm contraction.
[3]*Xenopus* Oocytes-Recombinant Adult-type Muscle nAChR
ND, no data

Key event No. 3: Sustained agonism of the foetal-type muscle nAChR and sustained muscle contracture

Toxicokinetic blood analyses across *in vivo* study types strongly suggests that blood concentrations of sulfoxaflor are maintained at steady-state levels due to continuous exposure *via* the diet. As sulfoxaflor readily perfuses into muscle from the blood (Rick et al., 2010) it is predicted that muscle sulfoxaflor concentrations would also be maintained at a steady state as long as dietary treatment continued. At the location of the NMJ nAChR, unlike ACh which undergoes tightly regulated synaptic vesicle release followed by rapid hydrolysis by acetylcholinesterase (AChE), sulfoxaflor

would remain at the nAChR synaptic cleft due to its lack of hydrolysis by AChE and thus receptor occupancy of sulfoxaflor would only be limited by association/dissociation kinetics of the molecule and not by removal from the receptor endplate region (as with ACh). Thus, upon foetal-type muscle nAChR activation, a sulfoxaflor-induced muscle contracture would be sustained for as long as sufficient sulfoxaflor molecules remain available for receptor binding, which is consistent with the observed experimental evidence.

- **Neonatal diaphragm**

To directly assess muscle contracture at the neonatal diaphragm, sulfoxaflor was tested for agonist action on the isolated phrenic nerve-hemidiaphragm preparation (Bulbring, 1946) from new-born rats. Since its introduction, the isolated phrenic nerve-hemidiaphragm preparation has become established as the standard nerve-muscle preparation for mechanistic investigations of drug action at the mammalian neuromuscular junction (see for e.g. (Liley and North, 1953; Hubbard and Wilson, 1973; Gibb and Marshall, 1984; Gibb and Marshall, 1986; Gibb and Marshall, 1987; Wareham et al., 1994; Fortier et al., 2001). The value of the preparation rests with the fact that it is amenable to both muscle tension and electrophysiological measurements and, as the main muscle involved in breathing (Vander et al., 2001), the preparation is routinely used to investigate responses of respiratory muscle (including impairment) to pharmacological test materials (e.g. muscle relaxant drugs used in surgery) (Gibb and Marshall, 1986; Gibb and Marshall, 1987; Bowman, 1990; Fortier et al., 2001). The phrenic nerve hemi-diaphragm experiments conducted with sulfoxaflor demonstrated a consistent, concentration-dependent contracture of the foetal-type diaphragm muscle (Figure B.6.6.12.9-8, Table B.6.6.12.9-7) and prolonged application of sulfoxaflor caused a sustained muscle contracture (Figure B.6.6.12.9-9). These experiments additionally demonstrated clear specificity as the contracture was completely reversible upon removal of sulfoxaflor. In addition, the sulfoxaflor-induced contracture was blocked by co-exposure with the highly selective muscle-type nAChR antagonist, tubocurarine, showing that the contracture induced by sulfoxaflor is mediated *via* nAChR activation, rather than a separate mechanism (more specifically, a post-receptor mechanism) (Figure B.6.6.12.9-8).

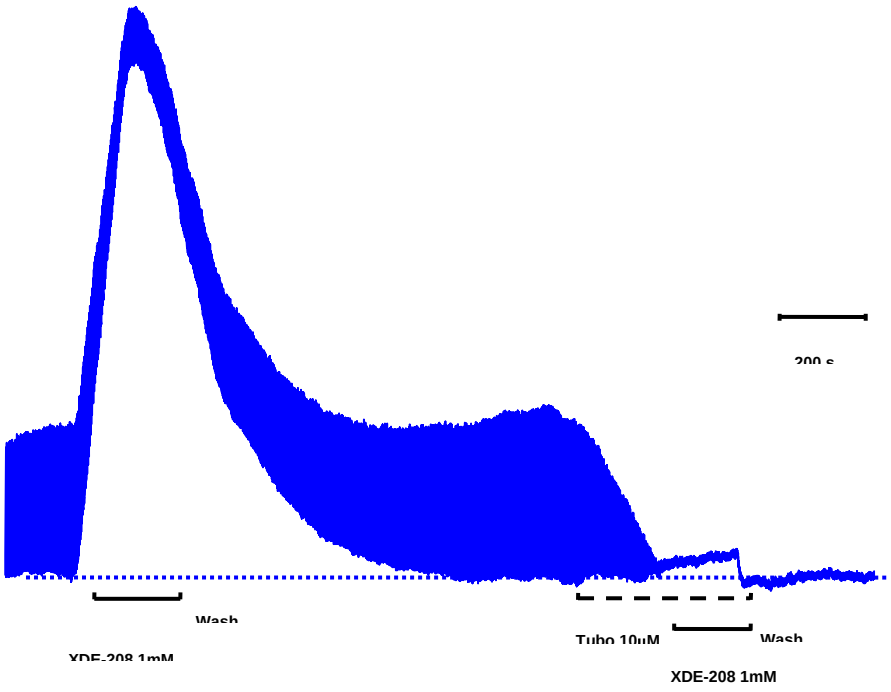


Figure 4.11.3.1.Study 8.8 (DAR Figure B.6.6.12.9-8.): Pre-application of 10µM tubocurarine (Tubo) effectively blocks the muscle twitches and antagonises responses to 100µM (not shown) or 1mM sulfoxaflor (Gibb, 2010)

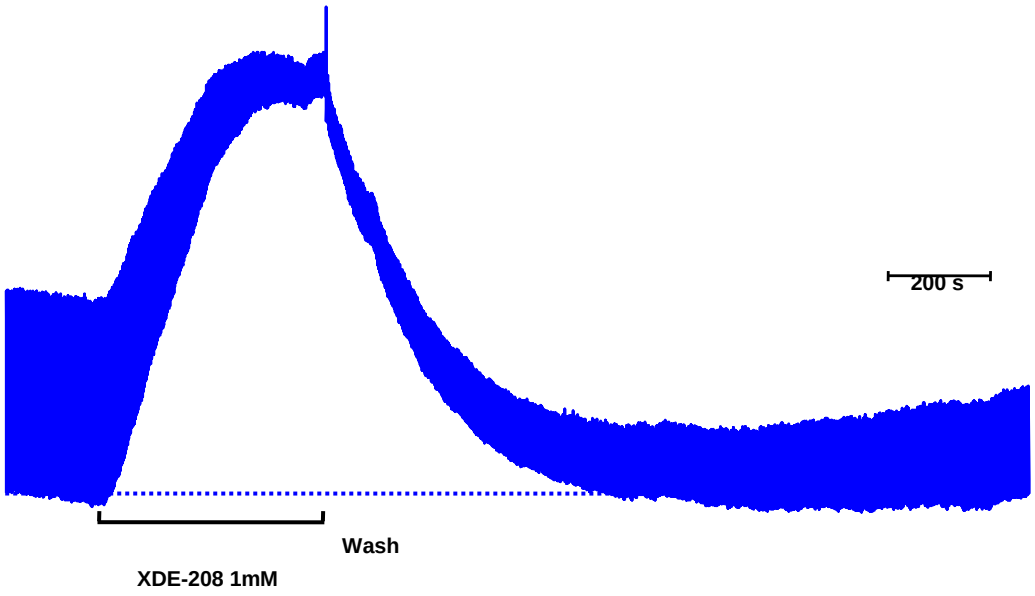


Figure 4.11.3.1.Study 8.9 (DAR Figure B.6.6.12.9-9.): Prolonged application (7 minutes) of 1 mM shows a sustained contracture by the diaphragm muscle (Gibb, 2010).

Sustained agonism *via* the muscle nAChR could potentially lead to contracture and dependent on dose, reduced responsiveness, or rigidity (Murray et al., 2009). A simplified version of the possible underlying chain of events following sustained activation of the muscle nAChR by sulfoxaflor can be summarised in the following steps: (1) depolarisation of muscle, (2) release of Ca++ from sarcoplasmic reticulum to muscle filaments, (3) elevated Ca++ levels in muscle filaments promote contraction, (4) muscle Ca++/ATPase membrane pump mediates muscle relaxation by reducing Ca++ levels in sarcoplasm, (5) sustained contraction reduces available ATP, (6) reduced ATP compromises active pump and Ca++ levels rise, (7) normal relaxation of the muscle cannot occur, and (8) contracture may occur (i.e., rigidity similar to *rigor mortis* but in live tissue).

b) Neonatal offspring limb contracture

The end result of this chain of events of muscle contracture is consistent with what was observed in rat foetuses at GD 21 C-section and in neonatal offspring shortly after birth (Table B.6.6.12.9-7). The forelimb flexure and hindlimb rotation observed in the guideline rat developmental toxicity and critical period studies (Tables B.6.6.12.9-4 and B.6.6.12.9-7) and the neonatal offspring death observed in the critical window and reproduction studies (Tables B.6.6.12.9-5 and B6.6.12.9-7) directly demonstrate a dose-dependent increase in sustained muscle contracture with increasing internal dose. In the assessment table for this key event (Table B.6.6.12.9-7) and the summary tables (Tables B.6.6.12.9-8 and B6.6.12.9-9) blood sulfoxaflor concentrations from the *in vivo*

studies have been converted from microgram per gram plasma to micromolar, to bridge *in vitro*, *ex vivo*, and *in vivo* data. While quantitative responses across these study types are not equivalent, this conversion facilitates qualitative comparisons of these responses for biological dose-response concordance. Using this approach *ex vivo* diaphragm muscle contracture is observed to occur at exposures which result in demonstrated *in vivo* developmental effects. Indirect evidence of the sustained agonism/sustained muscle contracture of the shoulder girdle was also evident with the skeletal finding of bent clavicle, a finding earlier discussed as dependent upon increased muscle contraction (Tables B.6.6.12.9-4 and B.6.6.12.9-7).

Table 4.11.3.1.Study 8.8 (DAR Table B.6.6.12.9-4.): Neonatal offspring limb and clavicle abnormalities resulting from sulfoxaflor induced sustained muscle contracture

	Critical Window 2[#]	**Critical Window 1[%]**	**Developmental Toxicity[^]**
Treatment Period	GD 20-22/LD 0	GD 16-Birth	GD 6-21
Applied Dose (PPM)	1000	1000	1000
Avg. TMI (mg/kg/day)	35.7	38.6	70.2
Internal Dose (µg/g)	D 5.41 – 16.1[A]	D 32.1 – 43.2	D 35.25 ± 5.4 F 30.00 ± 5.3
Forelimb Flexure[1]	P 7/96 (**7.3%**)[2] L 4/8 (50.0%)[2]	P 50/143 (**35.0%**))[2] L 11/12 (91.7**%**))[2]	F 122/295 (**41.4%**)) L 23/24 (95.8**%**))
Hindlimb Rotation	P 11/96 (**11.5%**))[2] L 6/8 (75.0**%**))[2]	P 19/143 (**13.3%**))[2] L 8/12 (66.7**%**))[2]	F 12/295 (**4.1%**)) L 7/24 (29.2**%**))
Bent Clavicle	P 0/86 (0**%**))[3] L 0/8 (0**%**))[3]	P 0/49 (0**%**))[3] L 0/7 (0**%**))[3]	F 40/133 (**30.1%**)) L 23/24 (70.8**%**))

[#] (Rasoulpour and Zablotny, 2010b), [%](Rasoulpour and Zablotny, 2010a), [^](Rasoulpour *et al.*, 2010d)
TMI = Test material intake
D = dam, F = foetus, L = litter, P = pup
A = three of the four sampled rats had undergone parturition prior to blood collection
N/A = not applicable
[1] A severe, >90° persistent flexure at the wrist or any flexure which cannot straighten
[2] Evaluated in surviving pups on PND 0
[3] Evaluated in surviving pups on PND 4

c) Neonatal offspring death

The incidence of the apical end point of neonatal death largely paralleled that of the limb and clavicle findings regarding its dose-response relationship (Figure B.6.6.12.9-2, Table B.6.6.12.9-5), consistency, and specificity across studies thereby supporting a single MoA for these effects.

Table 4.11.3.1.Study 8.9 (DAR Table B.6.6.12.9-5): Neonatal offspring death resulting from sulfoxaflor-induced sustained muscle

Study Type	Treatment Period	Applied Dose (PPM)	Avg. TMI (mg/kg/day)	Internal Dose (µg/g)	Incidence of Pup Death
Two Generation Reproduction[1]	Ten weeks prior to breeding – PND 21 (2 generations)	400	29.2	15.9 (LD 4)	4.5%
Reproduction Screening[2]	Two weeks prior to breeding – PND 21	500	30.3	N/A	18.8%
Critical Window 2[3]	GD 20-22/LD 0	1000	35.7	5.41 – 16.1[A]	10.4%
Critical Window 1[4]	GD 16-Birth	1000	38.6	32.1 - 43.2 (GD 21)	53.1%
Reproduction Screening[2]	Two weeks prior to breeding – PND 21	1000	62.0	14.3 - 41.9 (LD 4)	92.7%

TMI = Test material intake
A = three of the four sampled rats had undergone parturition prior to blood collection
1Rasoulpour et al., 2010e, 2Rasoulpour et al., 2010b, 3Rasoulpour and Zablotny, 2010b, 4Rasoulpour and Zablotny, 2010a

d) Reversibility of muscle contracture effects

The incidence of limb abnormalities decreased over the first two-to-four days of postnatal life. While a portion of the decreased incidence was due to pup deaths, reversibility of the limb abnormalities was directly demonstrated in the two critical window studies as evidenced by a 0% incidence of forelimb flexure and hindlimb rotation by PND 2 or 4 (Table B.6.6.12.9-6). Reversal of the bent clavicles was indirectly demonstrated by an absence of bent clavicles in PND 4 pups (of 86 pups evaluated in the affected groups), which is significant as these findings were observed at a relatively high incidence (30.1%) in GD 21 foetuses from dams exposed to 1000 ppm sulfoxaflor in the developmental toxicity study. These reversibility data suggest that the limb abnormalities were transient, consistent with a pharmacologic MoA for sulfoxaflor.

Table 4.11.3.1.Study 8.9 (DAR Table B.6.6.12.9-6.): Demonstration of reversibility of sustained agonism-induced limb contracture effects.

	Critical Window 1 Study[1]	Critical Window 2 Study[2]
Treatment Period	GD 16-Birth	GD 20-22/ LD 0
PND 0 to 1	11	5
PND 1 to 2	6	2
PND 2 to 3	3	0
PND 3 to 4	1	0
Total Reversals	**21**	**7**

1Rasoulpour and Zablotny, 2010a
2Rasoulpour and Zablotny, 2010b

An additional observation of sulfoxaflor's MoA specificity comes from the fact that *in vivo* definitive developmental toxicity and reproductive toxicity screening studies with X11719474 (the

insect inactive soil sulfoxaflor metabolite) showed no indication of these effects. Finally, there was a clearly demonstrated dose-response relationship as three *in vivo* studies showed clear lack of sustained muscle agonism at lower doses.

Table 4.11.3.1.Study 8.10 (DAR Table B.5.5.12.9-7.): Dose Response and Reversibility for MOA Key Event No. 3: Sustained Agonism and Sustained Muscle Contracture and Associated Death in Neonatal Offspring

Internal Dose (µM)	Applied Dose (ppm)	Key Event 3 Sustained Agonism & Sustained Muscle Contracture				Neonatal Apical Endpoints Forelimb Flexure, Hindlimb Rotation, Bent Clavicle, Death [&]
		Neonatal Diaphragm[$] (Foetal-type Muscle nAChR-dependent)	Reversible?	Neonatal Offspring Limb Contracture[^]	Reversible?	
2.3	25[1]			-		-
4.0	25[2]			-		-
ND	25[3]			-		-
15	150[1]			-		-
16	100[2]			-		-
ND	100[3]			-		-
57	400[2]			-		4.5
ND	500[3]			ND[+]		19
60	1000[5]			19	Y	19
89	1000[6]			ND[+]		100
100 µM *ex vivo*[7]		~10	Y			
101	1000[3]			ND[+]		93
108	1000[1]			76		76

Dose ↓

136	1000[4]			49	Y	53
1000 µM *ex vivo*[7]		100	Y			
X11719474 (a sulfioxaflorsoil metabolite inactive at the insect nAChR)						
244	5000[8]			-		-

[1]Rat Developmental Toxicity, [2]Two-Generation Reproduction, [3]One-Generation Reproduction, [4]Critical Phase 1 Mode of Action, [5]Critical Phase 2 Mode of Action, [6]Cross Foster, [7]Neonatal Diaphragm *Ex Vivo* Electrophysiology, [8]High dose level from the One-Generation Reproduction and Rat Developmental Toxicity studies.
[$]Estimated percent of maximum sulfoxaflor response
[^] Maximum incidence (foetal/pup basis) observed
[&] Maximum incidence (foetal/pup basis) observed
ND[+], No data as early studies did not examine this parameter
Y, Yes

Summary of Sulfoxaflor-induced Muscle Contracture and Death in Neonatal Rat Offspring MoA

This is a novel MoA assessment for sulfoxaflor-induced muscle contracture and death in neonatal rat offspring. The relevant molecular and apical endpoints for sulfoxaflor-induced developmental effects in rats are summarised in an abbreviated and full format in Tables B.6.6.12.9-8 and B.6.6.12.9-9, respectively. This analysis is based on the mechanistic and standard, repeat-dose developmental and reproductive toxicity studies in rats administered sulfoxaflor *via* the diet. Contextualisation of the dose-response relationship across study types (*in vivo, ex vivo, in vitro*) has been achieved through conversion of the *in vivo* internal dose metric (µg/g blood values) to micromolar units.

Key event No. 1 for the sulfoxaflor-induced muscle contracture and death in neonatal rat offspring is defined as binding to the foetal-type muscle nAChR. This event was assessed in competitive ligand binding assays with foetal-type nAChR preparations from GD 21 forelimb muscle tissue. Key event No. 2 for this developmental MoA is defined as agonism to the foetal-type muscle nAChR. This event was assessed by measuring agonist-induced ion fluxes through recombinant foetal and adult-type nAChRs expressed in *Xenopus* oocytes. Key event No. 3 for this developmental MoA is defined as sustained agonism at the foetal-type muscle nAChR and sustained muscle contracture. This event was assessed through a variety of experimental approaches including *ex vivo* phenic nerve hemidiaphragm preparations, where diaphragm muscle contracture was directly measured, and targeted *in vivo* MoA and standard guideline reproductive and developmental toxicity studies in which neonatal offspring limb and shoulder girdle contracture was observed. Supportive associative endpoints for this key event include the apical endpoints of forelimb flexure, hindlimb rotation, bent clavicle and neonatal death.

Table 4.11.3.1.Study 8.11 (DAR Table B.6.6.12.9-8.): Summary of Dose Response and Reversibility for MoA Key Events Related to Muscle Contracture and Associated Death in Neonatal Offspring (Abbreviated Table)

Plasma/ Media	Applied Dose	KE #1 Foetal-	KE #2 Foetal-	KE #3 Sustained Agonism & Sustained	Neonatal Apical

Conc. (µM)	(ppm)	type Muscle nAChR Binding[#]	type Muscle nAChR Agonism[$]	Muscle Contracture				Endpoints Forelimb Flexure, Hindlimb Rotation, Bent Clavicle, Death[&]
				Neonatal Diaphragm[$] (Foetal-type Muscle nAChR)	Reversible?	Neonatal Offspring Limb Contracture[^]	Reversible?	
Study Type		*In Vitro*	*In Vitro*	*Ex Vivo*		*In Vivo*		*In Vivo*
2.3-16 µM	25-150	-	-	-		-		-
30 µM		+	+	ND		ND		ND
57-89 µM	400-1000	ND				- to ++	Y	+ to +++
100-136 µM	1000	++	+ to ++	+	Y	+++	Y	+++
300-3000 µM		+++	+++	+++	Y	ND		ND

Percent inhibition of epibatidine binding: +, 0-25%; ++, 25-50%; +++, 50-100%
$ Percent of maximum XDE-208 response: +, 0-25%; ++, 25-50%; +++, 50-100%
^ Incidence (foetal/pup basis): +, 0-25%; ++, 25-50%; +++, 50-100%
& Maximum incidence (foetal/pup basis): +, 0-25%; ++, 25-50%; +++, 50-100%

Table 4.11.3.1.Study 8.12 (DAR Table B.6.6.12.9-9): Summary of Dose Response and Reversibility for MOA Key Events Related to Rat Muscle Contracture and Associated Death in Neonatal Offspring

Internal Dose (µM)	Applied Dose (ppm)	KE #1 Foetal-type Muscle nAChR Binding[#]	KE #2 Foetal-type Muscle nAChR Agonism[$]	KE #3 Sustained Agonism & Sustained Muscle Contracture				Neonatal Apical Endpoints Forelimb Flexure, Hindlimb Rotation, Bent Clavicle, Death[&]
				Neonatal Diaphragm[$] (Foetal-type Muscle nAChR)	Reversible?	Neonatal Offspring Limb Contracture[^]	Reversible?	
2.3	25[1]					-		-

Dose

4.0	25[2]					-		-
ND	25[3]					-		-
10 µM *in vitro*[4]		-	-					
15	150[1]					-		-
16	100[2]					-		-
ND	100[3]					-		-
30 µM *in vitro*[4]		+	+					
57	400[2]					-		+
ND	500[5]					ND[+]		+
60	1000[6]					+	Y	+
89	1000[7]					ND[+]		+++
100 µM *in vitro*[4]		++	+					
100 µM *ex vivo*[8]				+	Y			
101	1000[3]					ND[+]		+++
108	1000[1]					+++		+++
136	1000[5]					++	Y	+++
300 µM *in vitro*[4]		++	++					
1000 µM *in vitro*[4]		+++	+++					
1000 µM *ex vivo*[8]				+++	Y			
3000 µM *in vitro*[4]		+++	+++					
X11719474 (a sulfoxaflor soil metabolite inactive at the insect nAChR)								
3000 µM *in vitro*[4$]		ND	-					
244	5000[9]					-		-
Sulfoxaflor at the Adult-type Muscle nAChR								
3000 µM *in vitro*[4$]		ND	-					

[1]Rat Developmental Toxicity, [2]Two-Generation Reproduction, [3]One-Generation Reproduction, [4]Xenopus Oocytes-Recombinant Foetal-type Muscle nAChR, [5]Critical Phase 1 Mode of Action, [6]Critical Phase 2 Mode of Action, [7]Cross Foster, [8]Neonatal Diaphragm *Ex Vivo* Electrophysiology

[4$] Dose-response curve evaluated for muscle nAChR agonism (100-3000 µM).

[4*] Full dose-response data for foetal-type muscle nAChR binding and agonism showed in Table 3.

[9]Highest dose level from the One-Generation Reproduction and Rat Developmental Toxicity studies.

[#] Percent inhibition of epibatidine binding: +, 0-25%; ++, 25-50%; +++, 50-100%

[$]Percent of maximum sulfoxaflorresponse: +, 0-25%; ++, 25-50%; +++, 50-100%

[^] Incidence (foetal/pup basis): +, 0-25%; ++, 25-50%; +++, 50-100%

[&] Maximum incidence (foetal/pup basis): +, 0-25%; ++, 25-50%; +++, 50-100%

ND, No Data
ND^{+}, No data as early studies did not examine this parameter
Y, Yes

C. STRENGTH, CONSISTENCY, AND SPECIFICITY OF ASSOCIATION OF EFFECTS WITH KEY EVENTS

While strength, consistency and specificity of association were discussed within each key event assessment it will be briefly summarised herein (Table B.6.6.12.9-10). A thresholded, dose-dependent response was demonstrated within each key event across both applied and internal dose and this response was consistent across the key events, with no MoA-related effects at or below an applied dose of 100 ppm (three *in vivo* studies), thereby providing strong evidence of causality. With nine studies providing pertinent data, one of which being an *in vitro* and one being an *ex vivo* study, the association of effect was very robust (the seven *in vivo* studies had an R2 value of 0.91-0.93 across the dose-ranges tested). Specificity was demonstrated through sulfoxaflor's ability to completely, and dose-dependently, displace a high affinity nAChR ligand from nAChR binding sites. Also, the experiments presented confirm that compounds known to be negative for muscle contracture-induced limb abnormalities or neonatal death have little to no agonism at the foetal-type muscle nAChR (i.e. X11719474 and imidacloprid). Specific to sulfoxaflor, no agonism could be demonstrated at the adult-type muscle nAChR (even at the limit of solubility), consistent with a lack of contracture-related effects in adult rats *in vivo* at similar systemic sulfoxaflor levels. Specificity at the foetal-type muscle nAChR was further demonstrated by showing that sulfoxaflor-induced diaphragm contracture could be blocked by co-exposure with a nAChR-specific antagonist, tubocurarine, thereby ruling out sulfoxaflor action *via* a post-receptor mechanism.

Importantly, while agonism and muscle contracture was sustained in the presence of sulfoxaflor, without any evidence of receptor desensitisation or diminished agonism or muscle contracture response, its receptor-mediated pharmacologic agonist action in live affected offspring was rapidly reversible following sulfoxaflor's removal. This was demonstrated both in the *ex vivo* diaphragm contracture experiments and more importantly *in vivo* in live offspring with muscle contracture-related limb abnormalities. In conclusion, the *in vitro* binding and agonism data, the *ex vivo* diaphragm contracture data, and incidence of the apical end point of neonatal death largely paralleled that of the limb and clavicle findings regarding its dose-response relationship, consistency, and specificity across studies and thus support a single causal MoA for these effects.

D. BIOLOGICAL PLAUSIBILITY, TEMPORALITY, AND COHERENCE

The observed sulfoxaflor-induced limb and shoulder girdle contracture abnormalities and neonatal death are entirely biologically consistent with the functional ontogeny of the foetal-type muscle nAChR in the rat. In rats, the muscle nAChR develops functional subunit expression at the NMJ between GD 15 and 17 (Kues et al., 1995) resulting in synchronised foetal limb movements (Robinson and Smotherman, 1988) and diaphragmatic responsiveness between GD 16 and 17 (Bennett and Pettigrew, 1974), the latter being critical for the transition to extrauterine respiration. Functional expression of the γ (foetal-type) subunit continues through the first postnatal week and is largely complete by the end of the second postnatal week in limb and respiratory muscles (Missias et al., 1996). Importantly, this muscle receptor subtype is highly expressed during late gestation in the distal limbs muscles and diaphragm, with impairment of diaphragmatic maintenance of respiration at birth implicated in neonatal death from sulfoxaflor exposure.

Early critical window studies that were conducted in rats demonstrated that the critical period of developmental susceptibility to sulfoxaflor-induced offspring limb-contracture abnormalities and reduced survival was between GD 16 and 21 (Rasoulpour and Zablotny, 2010a) with follow-up

studies further narrowing this window to shortly before birth (GD 20-21) (Rasoulpour and Zablotny, 2010b). Furthermore, the cross-fostering study demonstrated that the neonatal offspring death requires prenatal exposure, but not postnatal sulfoxaflor exposure, indirectly providing support for the critical nature of proper foetal-type nAChR diaphragm function prior to birth (Rasoulpour and Zablotny, 2010c). Also supporting the biological plausibility of the hypothesis that sulfoxaflor acts *via* a foetal-type nAChR agonist-induced muscle contracture MoA is the observation that foetuses in the developmental toxicity study exhibited a hunched posture, consistent with generalised muscle contracture and had bent clavicles at the skeletal exam (Rasoulpour et al., 2010d), a finding consistent with the fact that the clavicle (collar) bone is highly-dependent upon muscle contraction for proper growth during the late foetal period, and alterations in muscle function having been demonstrated to affect clavicle development (Pai, 1965; Tran and Hall, 1989).

While this novel MoA is without clear published examples for which to address coherence of the MoA, the results from the conducted studies with sulfoxaflor, and related molecules, demonstrate adequately clear coherence of the biological effects (Table B.6.6.12.9-8). The finding that the primary effects of the reproduction and developmental studies (limb contracture abnormalities, bent clavicle, and neonatal offspring death) can all be aligned with agonist effects on muscle is important. While neonatal offspring death can result from a variety of complicating factors, the demonstration of diaphragm contracture *ex vivo* in the same strain and species and at exposure concentrations similar to those achieved *in vivo*, adds coherence to this MoA. The coherence of negative effects across end points and studies is also important to examine. As discussed earlier, exposure to a biologically inactive metabolite of sulfoxaflor, X11719474, resulted in no agonism at the foetal-type muscle nAChR and no developmental effects at 5-10 times the effect concentration of sulfoxaflor. Developmental toxicity and perinatal mortality studies have been conducted with sulfoxaflor in the New Zealand White rabbit and with both studies being negative for limb abnormalities or neonatal offspring death. In summary, these *in vivo* studies provide critical linkages of biological plausibility, temporality of association and, to the extent possible, coherence for this MoA.

Table 4.11.3.1.Study 8.13 (DAR Table B.6.6.12.9-10.): Analysis of Rat Muscle Contracture and Associated Death in Neonatal Offspring MoA for Sulfoxaflor (Bradford-Hill Criteria)

Key Event #1: Foetal-type muscle nAChR binding Key Event #2: Foetal-type muscle nAChR agonism Key Event #3: Sustained foetal-type muscle nAChR agonism and sustained muscle contracture Reversible effects (including *ex vivo* diaphragm and *in vivo* limb contracture data)	
Strength of association	+
Consistency of association	+
Specificity of association	+
Dose-response concordance	+
Temporal relationship	+
Coherence & plausibility	+ Plausibility; + Coherence

E. CONSIDERATION OF ALTERNATIVE MODE OF ACTIONS

In the process of evaluating and conducing experiments aimed at elucidating and testing the proposed MoA for sulfoxaflor, a number of alternative MoAs were ruled out. These included consideration of sulfoxaflor-induced limb abnormalities and neonatal offspring deaths due to: agonism at other AChR types (adult-type muscle nAChR, neuronal nAChR, muscarinic AChR), action downstream of the foetal-type muscle nAChR, antagonism at the foetal-type muscle nAChR, maternally-mediated foetal immobilisation, inhibition of acetylcholinesterase and inhibition of angiotensin-converting enzyme (Table B.6.6.12.9-11).

a) Agonism at other Acetycholine Receptor Types (Adult-type muscle nAChR, Neuronal nAChRs, Muscarinic AChR)

Adult-type nAChRs

The plausibility of sulfoxaflor's agonism at other ACh receptor subtypes was addressed through a combination of examination of the published literature and laboratory experiments. It has been previously proposed that foetal immobilisation due to uterine constraint (Gordon, 1998), which theoretically could result from contraction of maternal uterus may result in limb contracture effects in foetuses and offspring. This alternative MoA was directly addressed by examining the ability of sulfoxaflor to act as an agonist recombinant adult-type muscle nAChRs expressed in the previously described *Xenopus* oocyte system where up to sulfoxaflor's limit of solubility, no agonism was demonstrated. Additionally, the *in vivo* studies that demonstrated offspring effects, no clinical signs were observed which would be consistent with an effect on the adult muscle nAChR, such as muscle fasciculations or tonic or clonic limb contractions. The lack of agonism and agonist-associated clinical observations in these studies provides solid evidence to rule out agonism at the adult-type muscle nAChR as an MoA for sulfoxaflor's effects on the foetal limb contracture abnormalities and neonatal death.

Neuronal nAChRs

An alternative AChR in which to assess for plausability as an MoA is the potential action of sulfoxaflor at neuronal nAChRs. Theoretically this could be an operant MoA for the observed neonatal death, but not for the observed limb contracture abnormalities. Direct evaluations of sulfoxaflor agonism at individual neuronal nAChR subtypes have not been conducted because in the studies demonstrating developmental effects during sulfoxaflor exposure there have been no indications of neuronal nAChR-mediated clinical signs in the adults or offspring. A notable hallmark which follows neuronal nAChR activation is desensitisation of the receptors resulting in observations of muscle weakness or flaccid paralysis (Germiller et al., 1998). Of important distinction is that the sulfoxaflor-induced limb contractures consistently resulted in rigid, contracted limbs, further suggesting that neuronal nAChRs were not involved in the effects.

Foetal breathing movements are also known to be partially regulated by neuronal nAChRs in the brainstem. While respiratory rhythmogenesis is controlled primarily by neurons in the pre-Bötzinger complex through the neuromodulators (5-HT, substance P, catecholamines, high K+, and morphine), α4 and α7 containing neuronal nAChRs are present in the pre-Bötzinger complex and in motor nuclei innervating the respiratory muscles. The development of the respiratory rhythm generator must be well established and functionally robust by birth and foetal breathing movements are necessary for the proper maturation of the lungs with compounds which alter respiratory rhythm commonly resulting in lung hypoplasia (Dornan et al., 1984; Harding, 1995; Kobayashi et al., 2001). Notably, while prenatal nicotine exposure has been demonstrated to alter foetal lung development in rats (Maritz and van Wyk, 1997) and associated neuronal nAChR expression in primates (Sekhon et al., 1999) it paradoxically does not cause neonatal death during normoxic conditions in rats (Geller, 1959; Sobrian et al., 1995). In a histopathologic examination of GD 21

foetal rat lungs collected following sulfoxaflor maternal exposure no alterations were observed (Thomas and Marshall, 2010). This suggests that sulfoxaflor neonatal offspring death does not occur *via* a neuronal nAChR mechanism and is fundamentally different in its pharmacologic activity from nicotine, a molecule primarily active at neuronal nAChRs which causes neither neonatal death nor limb contracture abnormalities in rodents (Eugenin et al., 2008).

Muscarinic AChRs

Agonism at muscarinic AChRs has also been explored as an alternative MoA. As opposed to nAChRs which are ion channel receptors, muscarinic AChRs are G-protein coupled receptors of which there are 5 subtypes known with M1, M3 and M5 receptors having stimulatory and M2 and M4 receptors having inhibitory characteristics. The most plausible target by which a compound could cause neonatal offspring death would be *via* muscarinic activity at the mAChRs in the heart or lung. However, activity at these receptors would be accompanied by systemic clinical signs of mAChR activity and none of which have been observed in the studies presented. Also, gestation survival was unaffected, hearts were grossly normal on GD 21 visceral examination (including internal structures), and as discussed previously, lungs were histologically normal.

b) Agonism downstream of the foetal-type muscle nAChR

It is plausible that sulfoxaflor could exert its sustained muscle-contracture effects not directly at the nAChR but rather downstream of the receptor. This MoA (in adults) has been demonstrated with the herbicide, Cartap, which causes respiratory failure in adult rabbits (Liao et al., 1998) and marked irreversible contracture of adult-type mouse phrenic-nerve diaphragm preparations (Liao et al., 2000) in addition to its modulation of the neuronal nicotinic acetylcholine receptor in rats (Nagata et al., 1997). Rather than acting at the muscle nAChR directly, the diaphragm contracture MoA of cartap has been demonstrated be *via* post-receptor induction of extracellular Ca2+ influx, release of internal Ca2+, and an inhibition of [3H]ryanodine binding to the Ca2+ release channel of sarcoplasmic reticulum (Liao et al., 2000). In the phrenic-nerve hemidiaphragm experiments conducted with sulfoxaflor where sustained muscle contracture was demonstrated, a post-nAChR MoA would have been manifested by continued contraction in the presence of co-application with the nAChR antagonist tubocurarine. The sulfoxaflor-induced diaphragm contracture was completely eliminated with co-incubation with tubocurarine, thereby discounting a post-receptor MoA in the induction of the developmental effects of sulfoxaflor.

c) Foetal-type muscle nAChR inactivation

The most studied alternative MoA for decreased neonatal survival and neonatal limb abnormalities have come from studies in which the foetal-type muscle nAChR is either genetically absent or antagonised by various methods including: depolarising and non-depolarising blockade, autoimmune *myasthenia gravis*, or as previously discussed secondary desensitisation following neuronal stimulation. Knockout of the gamma subunit from the muscle nAChR results in decreased pre-natal and neonatal survival in gamma nAChR knockout mice (Takahashi et al., 2002), the forelimbs are functional while the hindlimbs are not, and in humans an inactive gamma subunit results in severe limb contractures, *pterygia* and increased intrauterine lethality (Michalk et al., 2008).

While knockout of the gamma subunit is an extreme example of nAChR inactivity, non-genetic, pharmacologic antagonism of the foetal-type muscle nAChR also results in neonatal offspring death and limb abnormalities. A historical example of this includes the use of tubocurarine as a non-depolarising neuromuscular blocking agent. Prenatal tubocurarine exposure during the last week of gestation in rats causes direct foetal paralysis capable of causing forelimb contracture, hindlimb

rotation and death (Shoro, 1977). While this may appear similar to the effects seen with sulfoxaflor there are several important distinctions. First of all, neuromuscular blocking agents do not readily cross the placenta (Evans and Waud, 1973). While this makes them particularly useful for pregnant women undergoing surgical operations, it is necessary to directly inject these agents into the foetus in order to exert these effects. Secondly, the limb contracture effects result in skeletal alterations in the limb cartilage, something that is not observed with sulfoxaflor. Finally, foetal paralysis additionally results in pulmonary hypoplasia, which has previously been mentioned to not occur following sulfoxaflor exposure.

Another historical example of agent-induced foetal paralysis resulting in neonatal death and limb contractures is plant alkaloid exposure in livestock. Examples include studies in cows, sheep, pigs, and goats following anabasine or conine exposure (Lee et al., 2006). Additionally, conine has been demonstrated to cause limb contractures in rabbits (Forsyth and Frank, 1993). There are two important findings that distinguish these agents from that of sulfoxaflor. Ultrasound studies using a goat model demonstrate decreased or eliminated foetal activity when the mother goat is fed anabasine (Weinzweig et al., 1999) that was associated with the observed muscle contractures, as well as cleft palate presumably resulting from inactive glossal muscle during palate closure. While the mode of action of the plant alkaloids on the foetus is not known, it could be speculated that the previously noted sedation or biphasic stimulation-depression seen in adults may be operant in the foetus and responsible for the inhibition of foetal movement. There are currently no published data for this supposition. Sulfoxaflor exposure did not result in cleft palate but did result in decreased offspring activity, presumably an effect of respiratory difficulties associated with a sustained contracture of the diaphragm muscle and other skeletal muscles. The other distinguishing finding of the alkaloids from sulfoxaflor is that a hallmark of plant alkaloid toxicity includes symptoms of acute cholinergic poisoning in the mother including fasiculations of muscle, clonic and tonic contractions of separate limbs, and convulsions followed by weakening and slowed heart rate, coma, and death (Bowman and Sanghvi, 1963). As discussed earlier these symptoms are consistent with neuronal nAChR stimulation and subsequent depression, which are not observed in either adults or neonates in the developmental and reproductive toxicity studies with sulfoxaflor in which developmental effects were observed. The best known plant alkaloid, nicotine, which causes increased prenatal death and no limb contractures in rodents, has a relative low adult LD_{50} due to its central convulsant action (Sheveleva et al., 1983; Sheveleva et al., 1984).

The last form of foetal-type muscle nAChR inactivation which will be discussed is foetal paralysis, and subsequent limb abnormalities induced by maternally-produced anti-AChR antibodies directed at functional foetal-type muscle AChRs. Blockade of ACh signaling at the foetal-type muscle nAChR causes multiple joint contractures that are associated with hypotonia, lung hypoplasia and perinatal death (Jacobson et al., 1999; Polizzi et al., 2000). As stated before, this MoA is not relevant to the findings in rats with sulfoxaflor as no hypotonia or lung hypoplasia was observed.

d) Acetylcholinesterase inhibition

Inhibition of acetylcholinesterase during pregnancy results in cholinergic signs of toxicity as previously discussed and ultimately result in maternal death (Farag et al., 2006). At sublethal exposure concentrations in rats cholinergic signs of AChE toxicity are generally observed as clinical signs in the absence of external, visceral, or skeletal abnormalities in foetuses, and thus do not fit the profile observed following sulfoxaflor exposure.

e) Angiotensin-converting enzyme (ACE) inhibition

Angiotensin-converting enzyme (ACE) inhibition presents another MoA that has been demonstrated to result in foetal limb contractures when exposure occurs during foetal development in humans

(Buttar, 1997). The limb contactures which occur are secondary to renal failure associated oligohydramniosis. Other secondary foetal anomalies including: potentially fatal hypotension, anuria, craniofacial deformities and hypoplastic lung development. In a few cases, postnatal persistence of a patent *ductus arteriosus* has also been linked to intrauterine exposure to ACE inhibitors (Barr, 1994). In contrast to humans, rodents are relatively resistant to the teratogenic effects of ACE inhibitors and high doses of ACE inhibitors typically result only in foetal growth retardation and occasionally increased pup death. Based upon the lack of similarity in neonatal abnormalities and the relative resistance of rodents to ACE inhibition-induced limb contractures this alternative MoA was considered not relevant to the findings with sulfoxaflor and was not considered further.

f) Conclusion of consideration of alternative MoAs

Following consideration of the presented alternative MoAs it is concluded that there is sufficient evidence to exclude these as plausible alternative MoAs for the observed neonatal offspring limb abnormalities and death. A summary evaluation for the considered alternative MoAs is presented in (Table B.6.6.12.9-11).

Table 4.11.3.1.Study 8.14 (DAR Table B.6.6.12-11.): Summary Evaluation for Other Possible MoAs in the Rat

Alternative MoA	Example	MoA Characteristics	Plaubility/ Coherence	Evidence
Adult-type NMJ nAChR agonism	Acetyl-choline	Muscle fasciculations, tonic or clonic limb contractions; Foetal immobilisation due to uterine contraction	- Plausibility/ Strong coherence across studies	Sulfoxaflor causes no agonism at the adult-type NMJ *in vitro*; no clinical signs in dev. tox. and repro. tox. studies
Neuronal nAChR subtype agonism	Nicotine, Epibatidine	Clinical signs of cholinergic stimulation followed by desensitization (inhibition); Lung hypoplasia	- Plausibility/ Strong coherence across studies	No indication of representative clinical signs in adults or offspring; normal lungs (including histopathology) in GD 21 foetuses
Muscarinic nAChR agonism	Muscarine, Carbamyl-choline	Clinical signs of cholinergic stimulation and/or inhibition; death caused by action at heart or lungs	- Plausibility/ Strong coherence across studies	No indication of representative clinical signs in adults or offspring; normal hearts (including internal structures) and lungs (incl. histopathology) in GD 21 foetuses
Agonism downstream of the foetal-type NMJ nAChR	Cartap	Muscle contracture associated with intracellular calcium or ATP regulation; not blocked by NMJ AChR antagonists	- Plausibility/ Limited coherence, only evaluated in *ex vivo* study	Diaphragm contracture completely blocked by co-exposure to the NMJ nAChR antagonist, α-tubocurarine
Foetal-type NMJ nAChR inactivation	α-Tubocurarine	Decreased or eliminated foetal activity; Limb contracture characterized by hypotonia; Altered limb skeletal structures; Lung hypoplasia	- Plausibility/ Good coherence across studies; Agents have limited placental transfer, requiring direct foetal injection	S-induced decreased activity likely due to respiratory difficulties; Limb tone and skeletal structures normal; Lungs normal
AChE inhibition	Neostigmine	Clinical signs of cholinergic toxicity resulting in	- Plausibility/ Strong coherence	No cholinergic clinical signs in adults or neonatal offspring

		maternal death; Sublethal exposures generally without	across studies	in dev. tox. and repro. tox. studies; no maternal death
ACE inhibition	Captopril, Enalapril	In humans, limb contractures secondary to renal failure associated oligohydramniosis; In rats, foetal growth retardation and occasionally increased pup death without limb contractures	- Plausibility/ Strong coherence across studies	Lack of similarity in neonatal abnormalities and resistance of rodents to ACE inhibition-induced limb contractures

F. UNCERTAINTIES, INCONSISTENCIES, AND DATA GAPS

a) Uncertainties

The limb and shoulder girdle contracture effects have been demonstrated to be reversible in live offspring. However, experimental amelioration or prevention of the effect has not been demonstrated to date.

b) Inconsistencies

In the 2-generation reproductive toxicity study, neonatal offspring limb contractures were not observed at doses which caused decreased pups survival. In addition, early studies (one-generation reproductive toxicity, dietary reproductive toxicity cross-fostering) did not examine this parameter.

c) Data Gaps

The limb and shoulder girdle contracture effects have been demonstrated to be reversible in live offspring. However, experimental amelioriation or prevention of the effect has not been demonstrated to date. Direct assessment of foetal-type nAChR inhibition or neuronal nAChR agonism by sulfoxaflor could be considered a data gap. Neither of these were conducted as they are inconsistent with the repeatable and robust observations in neonatal offspring for sulfoxaflor's developmental effects.

G. ASSESSMENT OF POSTULATED SULFOXAFLOR RAT MUSCLE CONTRACTURE MOA

The data for sulfoxaflor are judged with a high degree of confidence to adequately explain the induction of neonatal offspring limb contractures and death following sustained pharmacologic agonism at the foetal-type muscle nAChR by dietary sulfoxaflor exposure at the end of gestation in rats.

Based on the MoA analysis utilising the Bradford-Hill criteria for causality, there is a high degree of confidence that the observed sulfoxaflor-induced muscle contracture and associated death in neonatal offspring in rats occur *via* a single MoA through the following key events: (1) binding and (2) agonism at the foetal-type muscle nAChR by sulfoxaflor, thereby resulting in (3) sustained agonism and sustained muscle contracture in the foetus and neonatal pup. This sustained muscle contracture results in limb contractures, bent clavicles, and abnormal neonatal respiration after birth resulting in reductions in neonatal survival.

This novel MoA analysis demonstrates that the described MoA is plausible and has strong consistency, dose-responsiveness, and specificity across study types and dose ranges. The critical

period of sulfoxaflor's induced effects at the rat foetal-type muscle nAChR, and an absence of the effects in animals after PND 4, are consistent with this nAChR's ontogeny/maturational transitioning and spatial expression pattern which correlate with the timing and location of limb movement onset and foetal respiratory practice. The sulfoxaflor MoA analysis is summarised in Table B.6.6.12.9-10 regarding the criteria for the human relevance framework.

I. HUMAN RELEVANCE FRAMEWORK FOR SULFOXAFLOR-INDUCED RAT MUSCLE CONTRACTURE AND ASSOCIATED DEATH IN NEONATAL OFFSPRING

Question 1. Is the weight of evidence sufficient to establish the mode of action in animals? Based on this MoA analysis utilising the Bradford Hill criteria for causality, there is a high level of confidence that the observed sulfoxaflor-induced muscle contracture and associated death in neonatal offspring in rats occur *via* a singular MoA through the following key events: (1) binding and (2) agonism at the foetal-type muscle nAChR by sulfoxaflor, thereby resulting in (3) sustained agonism and sustained muscle contracture in the foetus and neonatal pup. This sustained muscle contracture results in limb contractures, bent clavicles, and abnormal neonatal respiration after birth resulting in reductions in neonatal survival.

This novel MoA analysis demonstrates that the described MoA is plausible and has strong consistency, dose-responsiveness, and specificity across study types and dose ranges. The critical period of sulfoxaflor's induced effects at the rat foetal-type muscle nAChR, and an absence of the effects in animals after PND 4, are consistent with this nAChR's ontogeny/maturational transitioning and spatial expression pattern which correlate with the timing and location of limb movement onset and foetal respiratory practice.

Question 2. Can human relevance of the MoA be reasonably excluded based on fundamental qualitative differences in key events between experimental animals and humans?

This MoA is considered not relevant to humans based upon data demonstrating fundamental qualitative differences in the agonism of sulfoxaflor at the rat or human foetal-type muscle nAChR. Specifically, binding but no agonism was evident with sulfoxaflor at the human foetal-type or human adult-type muscle nAChR (Figure B.6.6.12-10, Table B.6.6.12.9-13). Both muscle receptor types were examined as the transition from the foetal-type to adult-type human muscle nAChR occurs prenatally (Hesselmans et al., 1993). The species-specificity of the effects in the rat is further supported by the finding that although sulfoxaflor binds to the foetal rabbit muscle nAChR (Millar, 2010) is does not induce any developmental effects in this species despite similar systemic exposure. In conclusion, while sulfoxaflor demonstrates both clear binding and agonism to the rat foetal-type muscle nAChR, sulfoxaflor binds to, but does not induce any agonism to, the human foetal- or adult-type muscle nAChR. Furthermore, these findings would be expected to represent the human population as there are no known polymorphisms in the subunits which compose human muscle nAChRs.

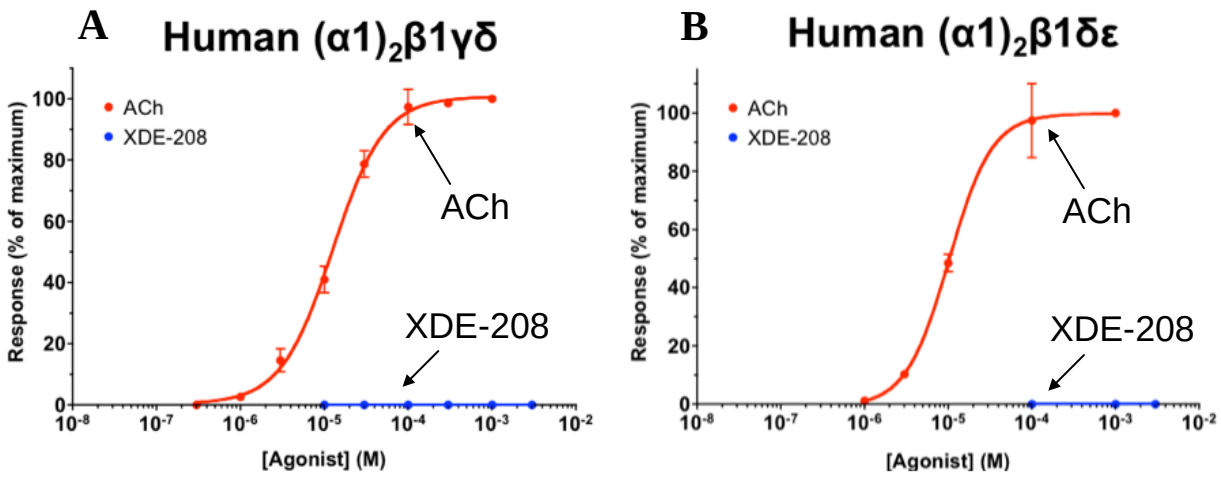


Figure 4.11.3.1.Study 8.9 (DAR Figure B.6.6.12.9-10.): Agonist activation of nAChRs expressed in *Xenopus* oocytes. Data are shown for the human foetal $(\alpha1)_2\beta1\gamma\delta$ nAChR (A) and human adult $(\alpha1)_2\beta1\delta\varepsilon$ nAChR (B). AChRs were expressed by microinjection of cDNA or cRNA in *Xenopus* oocytes. Dose-response curves are shown in which agonist-evoked responses are normalised to the maximal response detected with the endogenous agonist, acetylcholine (ACh). Data points are means of 3-7 responses. Adapted from Millar, 2010.

Table 4.11.3.1.Study 8.15 (DAR Table B.6.6.12.9-12.): Human Dose Response Data for MoA Key Events One and Two Related to Muscle Contracture and Associated Death in Neonatal Offspring

Internal Dose (µM)	Applied Dose (ppm)	Key Event 1 Human Foetal-type Muscle nAChR Binding[4,#]	Key Event 2 Human Muscle nAChR Agonism[5] Foetal | Adult	
3 µM *in vitro*		-	-	-
10 µM *in vitro*		+ (0.5%)	-	-
30 µM *in vitro*		ND	-	-
100 µM *in vitro*		++	-	-
300 µM *in vitro*		+++	-	-
1000 µM *in vitro*		+++	-	-
3000 µM *in vitro*		+++	-	-

10000 µM *in vitro*	+++	ND	ND

[4]Human embryonic kidney (HEK) cells expressing recombinant foetal-type muscle nAChRs
[#]Percent inhibition of maximum epibatidine binding: +, 0-25%; ++, 25-50%; +++, 50-100%
[5]Xenopus Oocytes-Recombinant Foetal- or Adult-type Muscle nAChR

Comparison of the amino acid sequence of the rat and human γ subunit revealed that, although the two subunits are similar (approximately 90% identical), they contain 53 amino acid differences (see Figure B.6.6.12.9-11) (Millar, 2010). There are several precedents for species-selective agonist activity of nAChR ligands. For example, nicotine is an agonist of neuronal α3β2 nAChRs from rat but is an antagonist of α3β2 nAChRs from chick (Hussy et al., 1994). Indeed, a single amino acid difference in the α3 subunit of rat and chick can account for the selective agonist effect of nicotine on rat and chick α3β2 nAChRs (Hussy et al., 1994). A similar situation has been reported for TMAQ, a nicotinic agonist that is selective for neuronal nAChRs containing a β4 subunit (Young et al., 2007). TMAQ binds to both human and rat α3β4 nAChRs but acts as an agonist only on human α3β4 nAChRs (Young et al., 2007). The selective agonist activity of TMAQ for human α3β4 nAChRs can be explained by two amino acids differences between the human and rat β4 subunit (Young et al., 2007).

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MHGGQGPLLLLLLLAVCLGAQGRNQEERLLADLMQNYDPNLRPAERDSDV    50
|||||||.|||||||.|||||.||||||||||||.||||.||||||||||
MHGGQGPQLLLLLLLATCLGAQSRNQEERLLADLMRNYDPHLRPAERDSDV    50

VNVSLKLTLTNLISLNEREEALTTNVWIEMQWCDYRLRWDPRDYEGLWVL    100
|||||||||||||||||||||||||||||||||||||||||||||||.|||||||.|
VNVSLKLTLTNLISLNEREEALTTNVWIEMQWCDYRLRWDPKDYEGLWIL    100

RVPSTMVWRPDIVLENNVDGVFEVALYCNVLVSPDGCIYWLPPAIFRSAC    150
||||||||.|||||.|||||||||||||||||||||||||||||||.|
RVPSTMVWQPDIVLGNNVDGVFEVALYCNVLVSPDGCIYWLPPAIFRSSC    150

SISVTYFPFDWQNCSLIFQSQTYSTNEIDLQLSQEDGQTIEWIFIDPEAF    200
||||||||||||||||.||||||||.||.||||||||.||||||||||
SISVTYFPFDWQNCSLVFQSQTYSTSEINLQLSQEDGQAIEWIFIDPEAF    200

TENGEWAIQHRPAKMLLDPAAPAQEAGHQKVVFYLLIQRKPLFYVINIIA    250
||||||||.|||||||||..||.|||||||||||||||||||||||||.
TENGEWAIRHRPAKMLLDPVTPAEEAGHQKVVFYLLIQRKPLFYVINIIV    250

PCVLISSVAILIHFLPAKAGGQKCTVAINVLLAQTVFLFLVAKKVPETSQ    300
||||||||||||.||||||||||||||.||||||||||||||||||||||
PCVLISSVAILIYFLPAKAGGQKCTVATNVLLAQTVFLFLVAKKVPETSQ    300

AVPLISKYLTFLLVVTILIVVNAVVVLNVSLRSPHTHSMARGVRKVFLRL    350
||||||||||||.|||||||||.||||||||||||||||||||||||||
AVPLISKYLTFLMVVTILIVVNSVVVLNVSLRSPHTHSMARGVRKVFLRL    350

LPQLLRMHVRPLAPAAVQDTQSRLQNG-SSGWSITTGEEVALCLPRSELL    399
|||||||||.|.|||||||...|||||.||||.|.|.|.||..|||||||||
LPQLLRMHVHPRAPAAVQDARLRLQNGSSSGWPIMTREEGDLCLPRSELL    400
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FQQWQRQGLVAAALEKLEKGPELGLSQ-FCGSLKQAAPAIQACVEACNLI   448
|.|.||.|||.|.|||||.|||...||.||||||||.|||||||.|||||.
FRQRQRNGLVQAVLEKLENGPEMRQSQEFCGSLKQASPAIQACVDACNLM   450

ACARHQQSHFDNGNEEWFLVGRVLDRVCFLAMLSLFICGTAGIFLMAHYN   498
|.|||||||||.|||||.||||||||||||||||||||||||||||||||
ARARHQQSHFDSGNEEWLLVGRVLDRVCFLAMLSLFICGTAGIFLMAHYN   500

RVPALPFPGDPRPYLPSPD   517
.||.||||||||||||.||
QVPDLPFPGDPRPYLPLPD   519
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Figure 4.11.3.1.Study 8.10 (DAR Figure B.6.6.12.9-11.): Sequence alignment of the rat and human nAChR γ subunits. An alignment is shown of the amino acid sequences of the human (top line) and rat (bottom line) nAChR γ subunits. Amino acids that are conserved between the two subunits are indicated by a vertical line (|). Amino acid differences are indicated by a dot (.). Gaps that have been inserted in one or other sequence in order to obtain an optimal alignment are indicated by a dash (-). Amino acids are indicated by standard one-letter abbreviations and are numbered from the first methionine (M) of the N-terminal signal sequence (Millar, 2010).

Given the evidence (described above) that one or two amino acid differences can confer species-selective agonist activity upon nicotinic ligands, it seems entirely plausible that the differences in agonist activity of sulfoxaflor can be explained by differences in the amino acid sequence of the rat and human nAChR γ subunits. The γ and ε subunits show even greater sequence differences that the human and rat γ subunit (even from the same species), where these subunits share only about 50% identity in amino acid sequence in the rat.

As there is no agonism of sulfoxaflor to the human foetal- or adult-type muscle nAChR there can be no sustained agonism or muscle contracture which operates by the demonstrated MoA in the rat.

Table 4.11.3.1.Study 8.16 Concordance of Key Events for Induction of Skeletal Muscle Contracture and Associated Death in Neonatal Offspring in Rats, Rabbits, and Humans.

Key Event	Evidence in Rats	Evidence in Rabbits	Evidence in Humans
#1 Binding to the foetal-type skeletal muscle nAChR	Yes, direct experimental evidence	Yes, direct experimental evidence	Yes, direct experimental evidence
#2 Agonism at the foetal-type skeletal muscle nAChR	Yes, direct experimental evidence	No data in rabbits, Not plausible given lack of developmental effects	No, direct experimental evidence of no sulfoxaflor agonism at the foetal- or adult-type skeletal muscle nAChR

#3 Sustained foetal-type skeletal muscle nAChR agonism/sustained muscle contraction	Yes, direct and indirect experimental evidence	No, direct and indirect experimental evidence	No data in humans. Not possible via this MoA as key event #2 does not occur.
Apical Endpoints Forelimb Flexure, Hindlimb Rotation, Bent Clavicle, Neonatal Death	Yes, direct experimental evidence	No, direct experimental evidence	No data in humans, Not possible *via* this MoA as key event #2 does not occur.

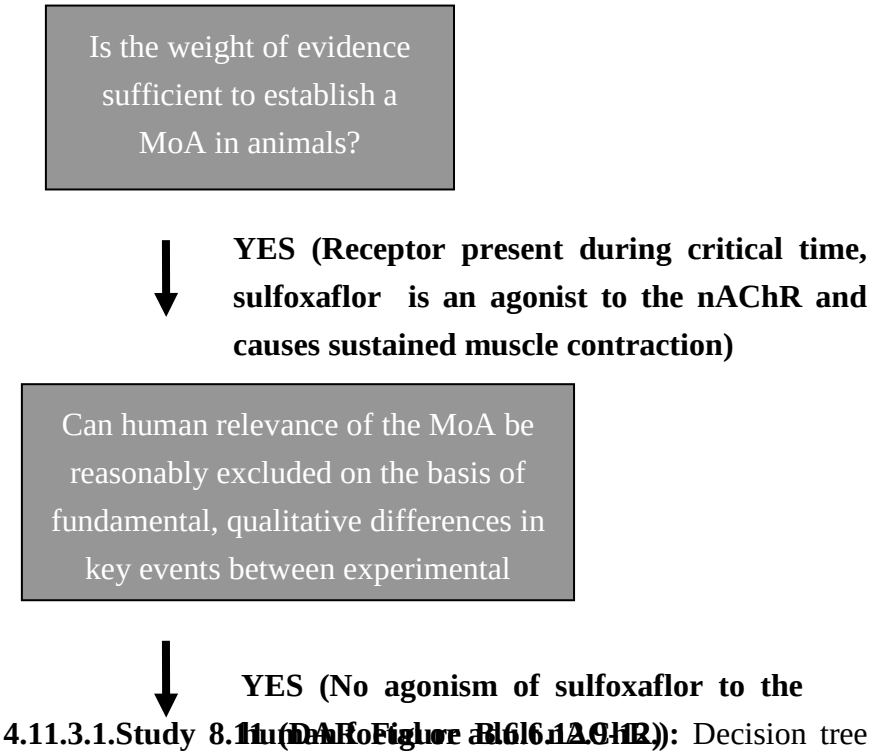

Figure 4.11.3.1.Study 8.11 (DAR Figure B6.6.12-12): Decision tree for determining human relevance of sulfoxaflor DART toxicity observed in MoA studies.

Question 3. Can human relevance of the MoA be reasonably excluded based on quantitative differences in either kinetic or dynamic factors between experimental animals and humans? As the human relevance of the experimental animal MoA was reasonably excluded on the basis of qualitative differences in key events (Question 2), a quantitative assessment of kinetic or dynamic factors is not necessary.

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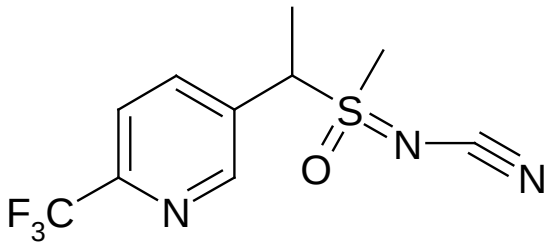
Data on Metabolites

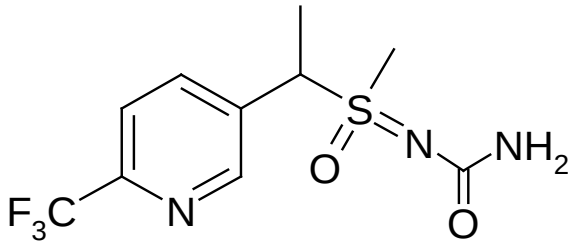
Sulfoxaflor has been demonstrated to be an agonist on rat foetal-type (α1β1γδ) skeletal muscle

nicotinic acetylcholine receptors (nAChRs). Two structurally related metabolites of sulfoxaflor (X11719474 and X11519540) were found to have no agonistic activity towards the rat foetal skeletal muscle nicotinic acetylcholine receptor and did not cause foetal abnormalities or neonatal death in rats. The inference is that these metabolites lack the functional group that enables binding or functional activation of the foetal receptor while being structurally very similar to the parent molecule sulfoxaflor.



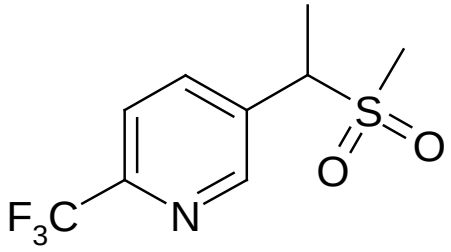
Parent: Sulfoxaflor

[methyl(oxo){1-[6-(trifluoromethyl)-3-pyridyl]ethyl}-λ4-sulfanylidene]cyanamide



Metabolite X11719474

N-(methyl(oxido){1-[6-(trifluoromethyl)pyridin-3-yl]ethyl}-λ4-sulfanylidene)urea



Metabolite X11519540

5-(1-methylsulfonyl)ethyl)-2-(trifluoromethyl)pyridine

Study 1: Rat reproduction / developmental screen for X11719474 (DAR B.6.8.1.8.1)

Report:	R. J. Rasoulpour, A. K. Andrus, R. G. Ellis-Hutchings, and B. L. Yano. (2010e). X11719474: Dietary Reproduction/Developmental Toxicity Screening Test in CRL:CD(SD) Rats. Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan, 48674. Study ID: 081153 (29 January 2010). MRID 47832094. Unpublished.
Dates of work:	October 2, 2008 to January 22, 2010
Guidelines:	USEPA OPPTS 870.3550 (2000) OECD Guideline No. 421 (1995)
GLP/Compliance:	Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were provided.

Executive Summary: Groups of 12 male and 12 female Crl:CD(SD) rats were administered the sulfoxaflor metabolite X11719474 *via* the diet at concentrations supplying 0, 1000, 2000, or 5000 ppm, which corresponded to time-weighted average doses of 0, 80.8, 162, or 396 mg/kg/day for males, and ranged from 0, 81.7-114, 167-212, and 451-507 mg/kg/day during the female pre-breeding, gestation and lactation phases. Males were fed the test diets for two weeks prior to breeding and continuing through breeding (up to two weeks) up until necropsy (test day 39). Females were fed the test diets for two weeks prior to breeding, through breeding (up to two weeks), gestation and lactation up until necropsy on post-partum day 22-24. Effects on gonadal function, mating behavior, conception, development of the conceptus, parturition, and postnatal growth and survival were evaluated. In addition, a gross necropsy and histopathologic examination of the adults were conducted with an emphasis on organs of the reproductive system. Offspring were evaluated through postnatal day (PND) 21 for litter size, survival, sex, body weight and the presence of gross external morphological alterations. Plasma concentrations of X11719474 were measured in PND 4 culled pups to demonstrate systemic exposure.

There were no treatment-related clinical findings or adverse effects on feed consumption at any dose level. Body weight and/or body weight gain in the 2000 and 5000 ppm groups were slightly lower than controls during isolated gestation and lactation intervals. However, these findings were considered of no toxicological significance based on the lack of a dose-response relationship, the small magnitude of change, and lack of consistency across intervals.

There was a treatment-related increase in the liver weights of males and females (15 and 7% increase in relative weight, respectively) in the 5000 ppm group. These findings corresponded with an increased incidence of very slight centrilobular and midzonal hepatocellular hypertrophy in all males and the majority of females (10/12) in the 5000 ppm group. There were no treatment-related organ weight or pathologic effects in the 1000 and 2000 ppm groups of either sex. Toxicokinetic data demonstrated dose-proportional systemic exposure in PND 4 pups.

There were no treatment-related effects on reproductive endpoints or development of the offspring at any dose level.

There was slight maternal toxicity at the highest dose tested of 5000 ppm (396 mg/kg/day); therefore the NOAEL for maternal toxicity was 2000 ppm (162 mg/kg/day).

There was no indication of reproductive or developmental toxicity at the highest dose tested of 5000 ppm (396 mg/kg/day); therefore the reproductive and developmental NOAEL was 5000 ppm (396 mg/kg/day).

This study was fully reliable (acceptable/guideline) and satisfies the guideline requirements for a dietary screening reproduction/developmental study in rats (OPPTS 870.3550; OECD 421).

Study 2: Rat prenatal developmental study for X11719474 (DAR B.6.8.1.8.2)

Report:	Rasoulpour, R. J. and Marshall, V.A. (2010f). X11719474: Dietary Developmental Toxicity Study in CRL:CD(SD) Rats. Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan, 48674. Study ID: 081043, (23 February 2010). MRID 47832087. Unpublished.
Guidelines:	USEPA OPPTS 870.3700 (1998) OECD Guideline No. 414 (2001) EEC, ISSN 1725-2555 (2004) JMAFF Guideline 2-1-18, Teratogenicity Study (2000)
Dates:	April 12, 2009 (study initiation) – May 12, 2009 (end experiments)

GLP: Yes (certified laboratory).
Signed and dated GLP, Quality Assurance, Flagging and (No) Data Confidentiality statements were provided

Summary: In a dietary developmental toxicity study (MRID 47832087), groups of 26 time-mated female CD rats were given the sulfoxaflor metabolite X11719474 (purity 99.5% a.i. wt/wt, Lot # E2695-1, TSN030626-0003) in feed at concentrations of 0, 1000, 2000, or 5000 ppm on gestation days (GD) 6 through 21, which corresponded to time-weighted average doses of 0, 74, 152, or 368 mg/kg/day, in order to evaluate the maternal and developmental toxicity potential of this compound.

Treatment-related effects were limited to a transient decrease in body weight gain in the 5000 ppm group at the initiation of treatment, with a concomitant decrease in feed consumption. This finding was deemed of no toxicological significance as it was minor in nature, isolated to the first three days of treatment, and likely due to decreased palatability of the diet. There was no treatment-related maternal toxicity at 1000 or 2000 ppm. There was no treatment-related organ weight or gross pathologic findings at any tested dose level. Toxicokinetic analyses of dam and fetal plasma X11719474 concentrations revealed dose proportionality across all groups and concentrations that were similar in dams and fetuses from the same groups. There was no evidence of developmental toxicity at any dose level tested in this study.

There was no indication of maternal or developmental toxicity at the highest dose tested; therefore , under the conditions of this study, the no-observed-adverse-effect level (NOAEL) for maternal and developmental toxicity was ≥ 5000 ppm (368 mg/kg/day).

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The developmental toxicity study in the rat is fully reliable (acceptable/guideline) and satisfies the guideline requirement for a Prenatal Developmental Toxicity Study (OPPTS 870.3700; OECD 414) in rats.

Study 3: Rat reproduction / developmental screen for X11519540 (DAR B.6.8.5.6.2)

Report: Rasoulpour, R. J., Zablotny, C. L., McCoy, A. T., and Thomas, J. (November 21, 2011). X11519540: Dietary Reproduction/Developmental Toxicity Screening Test in Crl:CD(SD) Rats. Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan, 48674. Study ID: 111040.
Guidelines: USEPA OPPTS 870.3550
OECD Guideline No. 421
Dates: November 2011
GLP: Yes (certified laboratory).
Signed and dated GLP, Quality Assurance, Flagging and (No) Data Confidentiality statements were provided

Ten male and ten female Crl:CD(SD) rats per group were administered 0, 25, 100, or 300 ppm X11519540 in the diet, which resulted in time-weighted average doses of 0, 2.03, 8.11 or 23.4 mg/kg/day for males and 0, 2.01-2.11, 7.69-8.88, or 23.4-27.1 mg/kg/day for females, respectively. Males were administered test diets for at least two weeks prior to breeding and continuing through breeding for 29 days. Females were given test diets for two weeks prior to breeding, through breeding (up to two weeks), gestation (three weeks), and lactation (three weeks). Effects on gonadal function, mating behavior, conception, development of the conceptus, parturition and early postnatal growth and survival were evaluated. In addition, a gross necropsy and histopathology of the adults was conducted with an emphasis on organs of the reproductive system. Plasma samples were collected from adult males on TD 15, from adult females on LD 4,

and from culled pups on PND 4 for analysis of X11519540 concentration. In the offspring, litter size, pup survival, sex, body weight, and the presence of gross external morphological alterations were also assessed.

Treatment-related effects on body weight and feed consumption were limited to males in the 300 ppm group that consisted of non-statistically significant reductions in feed consumption from TD 1-5 and decreased body weight on TD 22 and 29, relative to controls. Males and females in the 300 ppm group had treatment-related increases in absolute and relative liver weights (48.7% and 57.3%, and 25.7% and 21.4%, respectively). In addition, males in the 100 ppm group also had treatment-related increases in absolute and relative liver weights (28.6% and 32%, respectively). These increased liver weights corresponded to treatment-related very slight-moderate hepatocellular hypertrophy accompanied by altered cytoplasmic tinctorial properties. A small proportion of males in the 300 ppm group also had a very slight increased incidence of mitotic hepatocytes.

In addition to liver effects, there were minor treatment-related effects in the kidneys, adrenal glands, and thyroid glands. Males and females given 300 ppm had a very slight increase in cytoplasmic vacuolization of the zona glomerulosa of the adrenal cortex. The thyroid glands of males given 100 and 300 ppm showed a very slight hyperptrophy of the follicular epithelial cells. A very slight follicular cell hypertrophy was also observed in a small proportion of females given 300 ppm. Finally, males in the 100 and 300 ppm groups had treatment-related effects in kidney weight with no histopathological correlating changes.

Toxicokinetic analysis determined that X11519540 was present at levels above the analytical lower limit of quantitation in all plasma samples collected from adult males, dams and pups. Concentrations of X11519540 were similar between adult males, dams and male and female pups, suggesting no sex-related differences in the kinetics of X11519540 and that X11519540 freely partitions into the milk. Plasma concentrations in all four treatment groups were less than dose-proportional at 300 ppm. Plasma concentrations in female pups were also less than dose-proportional at 100 ppm.

Dietary exposure to X11519540 resulted in no indication of reproductive toxicity at any dose level tested. There were no adverse effects on prenatal/early neonatal growth or survival.

Under the conditions of this study, the no-observed-effect level (NOEL) for slight systemic toxicity was 25 ppm, the NOEL for reproductive toxicity was 300 ppm, the highest dose level tested.

This study is acceptable and satisfies the guideline requirement for a Dietary Reproduction/Developmental Toxicity Screening Test -- Rats (OPPTS 870.3550, OECD 421).