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ISOFLUCYPRAM

Volume 3 – B.6 (AS)

Rapporteur Member State : United Kingdom
Co-Rapporteur Member State : France

Version History

When	What
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B.6. TOXICOLOGY AND METABOLISM DATA

Isoflucypram (CAS-No. 1255734-28-1) is a new fungicidal active substance developed by Bayer for application on cereals.

It is a novel broad-spectrum fungicide of the chemical class of N-cyclopropyl-N-benzyl-pyrazole-carboxamides and a succinate dehydrogenase inhibitor (SDHI) in fungi. Succinate dehydrogenase is also present in mammals and humans – whether isoflucypram is capable of inhibiting this enzyme in humans is not know; however, it is noted that isoflucypram is extensively metabolised and rapidly eliminated in rats (see section B.6.1 below).

Isoflucypram representative product (ISY EC 50) is an emulsifiable concentrate (EC) containing 5% isoflucypram.

As this is a new substance, there is no information on its toxicity in publicly available literature (see section B.6.10 of document CA_B6). The available regulatory studies were conducted for the purpose of this approval and have not been previously evaluated in the EU; the studies were GLP-compliant and followed the respective OECD test guidelines.

This document uses the term "isoflucypram" when referring to the active substance. However, other synonyms (development codes) may have been used by the applicant within the individual study reports: BCS-CN88460, '460' and ISY.

The batches of isoflucypram used in the toxicology studies are considered representative of the technical specification (see Vol 4 for more details).

The majority of the methods of analysis for the active substance in different matrices (diet, air, gavage solutions) used in the *in vivo* toxicological studies are either validated or fit for purpose (see document CA_B5 and individual studies within this B6 document for further details).

The human health classification of isoflucypram has been addressed in an aligned CLH dossier submitted to ECHA.

The data requirements of regulation (EC) 1107/2009 and Reg 213/2013 have been met and the RMS concludes that there are no data gaps.

B.6.1. Absorption, distribution, metabolism and excretion in mammals

The toxicokinetics of isoflucypram have only been investigated following oral administration in rats. Some further limited information is available from dietary repeated dosing toxicity studies in rats, mice and dogs and from a rat developmental toxicity study. In addition, a comparative *in vitro* metabolism study is also available.

Five oral dosing studies in rats are available (four with isoflucypram and one with the metabolite M12): ADME studies with ^{14}C pyrazole and ^{14}C phenyl radiolabeled isoflucypram, quantitative whole-body autoradiography studies with ^{14}C phenyl and ^{14}C pyrazole radiolabeled isoflucypram and a study with the main carboxylate metabolite (M12). These studies followed standard OECD test Guidelines and were GLP compliant. Additional limited toxicokinetic information from repeated dose studies conducted in rats, mice and dogs and from the rat developmental toxicity study is also included in this section. The RMS considers these studies to provide a thorough understanding of the toxicokinetics of isoflucypram in experimental animals following oral dosing.

B.6.1.1. Absorption, distribution, metabolism and excretion by oral route

Study 1: ADME – ^{14}C pyrazole labelled isoflucypram

Study	Amendment no 1 to final report - [Pyrazole-4-14C]BCS-CN88460 - Absorption, distribution, excretion and metabolism in the rat
Reference	[redacted] (2017a)
Date performed	21/09/2017

Test facility	[redacted]
Report reference	M1824604-5
Guideline(s)	OECD 417
Deviations from the guideline	None
GLP	Yes
Test material	^{14}C pyrazole – 98.4% (radiochemical purity >98%)
Study acceptable	Yes

Methods

The toxicokinetic behaviour of isoflucypram labelled with ^{14}C in the pyrazole-4 moiety, was investigated in male and female Wistar rats in groups of between four and eight animals. Seven experiments were conducted in this study comprising low dose (2 mg/kg bw) and high dose (200 mg/kg bw) isoflucypram and low dose repeated treatment. The design for each experiment is set out in table B.6.1-1. The rats received radiolabelled isoflucypram by oral gavage as a suspension in water and tragacanth at pH 4. They were sacrificed three days post dosing and in the case of bile-duct cannulation two days post dosing. The total radioactivity included the radioactivity related to the test compound and the metabolites and was determined in plasma, urine, bile, faeces, organs and tissue samples at sacrifice. Metabolites were investigated in urine, bile and extracts of faeces. In this study. TOPFIT version 2.0 was used to calculate toxicokinetic parameters by plasma concentration-time curve analysis and a standard 2-compartment disposition model was applied for curve fitting computation.

Figure B.6.1-1: Isoflucypram, showing position of radiolabel*

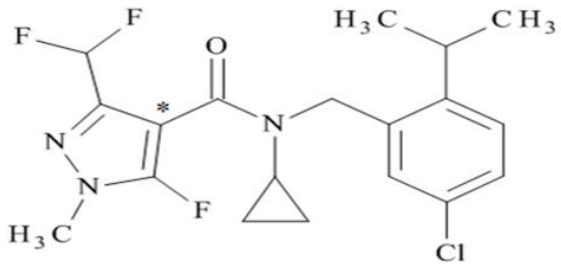


Table B.6.1-1: Study Design

Experiment	**Numbers/Dose/Dose regimen**	**Time of sample collection**				
		Urine [h]	**Faeces** [h]	**Bile** [h]	**Organs** [h]	**Microplasma** [h]
1	4 male rats 2 mg/kg bw single oral low dose	4, 8, 12, 24, 48, 72	24, 48, 72	---	72	0.25, 0.5, 1, 2, 7, 24, 48, 72
2	4 female rats 2 mg/kg bw single oral low dose	4, 8, 24, 48, 72	24, 48, 72	---	72	
3	4 male rats 200 mg/kg bw single oral high dose	4, 8, 24, 48, 72	24, 48, 72	---	72	

4	4 female rats 200 mg/kg bw single oral high dose	4, 8, 24, 48, 72	24, 48, 72	---	72	0.25, 0.5, 1, 2, 4, 7, 24, 48, 72
5	4 male rats 2 mg/kg bw low dose pre-treatment with non-radioactive test compound for 14 days plus a single radioactive low dose	4, 8, 24, 48, 72	24, 48, 72	---	72	
6	6 bile-duct cannulated male rats * 2 mg/kg bw single oral dose	4, 8, 24, 48	24, 48	4, 8, 24, 32, 48	48	---
7	8 bile-duct cannulated female rats ** 2 mg/kg bw single oral dose	4, 8, 24, 48	24, 48	4, 8, 24, 32, 48	48	---

* Two animals were sacrificed approx. 24 h after treatment, due to no bile being collected.
** Four animals were sacrificed during the test or not used for evaluation

Results

Recovery was good with between 100.3% and 104.5% of the administered radioactivity being recovered at sacrifice, for all experiments

Absorption

Radioactivity was detected in plasma at 0.25h and the maximum plasma concentration (Cmax) was reached within 1 h (tmax) post administration for the low dose tests (2 mg/kg bw) for both, males and females. In the high dose tests with 200 mg/kg bw, the mean maximum plasma concentration was reached at 2 h post-dosing for males and at 4 h post-dosing for females. The time course of the mean plasma levels was comparable in male and female rats in all tests.

Table B.6.1 -2: Recovery of radioactivity in excreta, gastrointestinal tract and the carcass of rats following oral dosing of [pyrazole-4-^{14}C]isoflucypram - percent of total radioactive administered dose (mean values)

Test no.	**Test 1**	**Test 2**	**Test 3**	**Test 4**	**Test 5**	**Test 6**	**Test 7**
	male	**female**	**male**	**female**	**male**	**male**	**female**
Experiment	**single low dose**	**single low dose**	**single high dose**	**single high dose**	**Repeated single dose**	**bile-duct cannulation**	**bile-duct cannulation**
Dose	**2 mg/kg bw**	**2 mg/kg bw**	**200 mg/kg bw**	**200 mg/kg bw**	**2 mg/kg bw**	**2 mg/kg bw**	**2 mg/kg bw**
Urine	14.06	12.00	7.11	8.51	12.67	5.89	2.37
Bile	---	---	---	---	---	77.61	85.45
Faeces	89.28	88.14	93.44	92.03	90.01	20.67	15.52
Total excreted	**103.34**	**100.13**	**100.55**	**100.54**	**102.68**	**104.16**	**103.34**
Body excluding GIT	0.379	0.172	0.153	0.155	0.294	0.252	0.218
GIT	0.030	0.016	0.017	0.012	0.045	n.c.	0.980
Total in body	0.409	0.188	0.170	0.167	0.339	0.306	1.198

Balance	**103.75**	**100.32**	**100.72**	**100.71**	**103.02**	**104.47**	**104.54**

In bile duct cannulated rats, the % of the administered dose recovered in bile after 4-hours was 36-43% (mean 40%) in males and 21-58% (mean 35.44%) in females, rising to 69-86% (mean 77.01%) and 73-93% (mean 82.25%) in males and females respectively after 24-hours. These data indicate that biliary excretion of the single low dose of isoflucypram is rapid and essentially complete after 24 hours in rats.

It is also noted that the amount recovered in the urine, in low dose non bile duct cannulated animals is around 8-10% higher than in bile-duct cannulated animals, indicating some enterohepatic recirculation.

Oral absorption has been calculated by summation of the recovered radioactivity in urine, bile, and body without gastrointestinal tract -GIT (83.75 and 88.04% in males and females respectively) from the single low dose experiment. The calculated values indicate that oral absorption is high at the low dose. However, as a significant amount of the administered dose is excreted in bile within the first 24 hours (76-82%), a value for post-hepatic systemic availability has also been derived, by excluding the material in the bile. Post-hepatic systemic availability has been calculated by summing the recovered radioactivity in urine and in the body minus GIT in low single dose animals and accounts for 12 and 14.4% of the administered dose, in males and females respectively (see tables B.6.6-3 and B.6.6-4 below). Overall, an **oral absorption** value of **100%** and a **post-hepatic systemic availability value of 15%** can be determined from the available data.

Table B.6.1-3: Oral Absorption

Source	**Male** **bile-duct cannulation** **2 mg/kg bw**	**Female** **bile-duct cannulation** **2 mg/kg bw**
Urine (%)	5.89%	2.37%
Bile (%)	77.61%	85.45%
Body minus GIT (%)	0.252%	0.218%
Absorption from GIT (%)	**83.75%**	**88.04%**

Table B 6.1-4: Post Hepatic Systemic Availability

Source	**Male** **2 mg/kg bw**	**Female** **2 mg/kg bw**
Urine (%)	14.06%	12.00%
Body minus GIT (%)	0.379%	0.172%
Post Hepatic Exposure (%)	**14.4%**	**12%**

Distribution

At sacrifice low levels of radioactivity (between 0.153% and 0.379% of the recovered dose) were found in the carcass excluding the GIT, at both dose levels, indicating that almost all the administered dose was eliminated with none retained in the carcass. Low amounts of radioactivity were detected in the GITs (0.012% to 0.045 %), excluding GITs from bile-duct cannulated rats. These data indicate that low levels of the administered dose were retained in the carcass. At the end of the study, radioactivity levels in blood in any dose group were below 0.1% of the administered dose. Individual metabolites were not identified in blood.

The highest concentration of radioactivity was detected in the liver and ranged from 0.05 to 0.2% of the administered dose. The highest levels were observed in low-dose males. The concentration in red blood cells (around 0.01-0.02% of the administered dose) was relatively high compared to the concentrations in organs and tissues excluding liver (no higher than 0.005% of the administered radioactivity).

Table B 6.1-5 Radioactive residues in organs and tissues at sacrifice expressed as % dose administered

Organs/ Tissues	Test 1 male 2 mg/kg bw	Test 2 female 2 mg/kg bw	+Test 3 male 200 mg/kg bw	Test 4 female 200 mg/kg bw	Test 5 repeated male 2 mg/kg bw
Blood Cells**	0.0246	0.0133	0.0137	0.0145	0.0142
Plasma**	0.0117	0.0046	0.0034	0.0091	0.0053
Carcass	0.0808	0.0523	0.0387	0.0488	0.0650
Heart	0.0011	0.0006	0.0005	0.0007	0.0010
Brain	0.0007	0.0004	* 0.0003	0.0005	0.0005
Kidneys	0.0053	0.0026	0.0023	0.0020	0.0045
Liver	0.1969	0.0659	0.0675	0.0568	0.1597
Testes	0.0020	---	0.0008	---	0.0018
Ovaries	---	0.0001	---	* 0.0001	---
Uterus	---	0.0005	---	n.c.	---
Adrenal gland	0.0001	0.0001	* <0.0001	0.0001	0.0001
Thyroid gland	n.c.	n.c.	n.c.	n.c.	n.c.
Spleen	0.0006	0.0004	0.0003	0.0005	0.0005
Lung	0.0032	0.0013	0.0013	0.0019	0.0035
Eyes	0.0001	0.0001	0.0001	0.0001	0.0001
Skin	0.0494	0.0279	0.0232	* 0.0195	0.0365
Bone femur**	0.0003	n.c.	n.c.	n.c.	0.0004
Perirenal fat**	0.0007	0.0014	0.0003	0.0005	0.0002
Muscle leg**	0.0011	0.0005	0.0005	0.0006	0.0008

*- mean value calculated with half of LLQ (Lower Limit Quantification)
**- Of these organs or tissues only an aliquot was sampled at sacrifice, the percentage of dose Administered is relating to the aliquot of organ/tissue sampled and analysed. The contribution of The part which was not sampled is included in the value for the carcass.

From peak levels, the time course of radioactivity in plasma showed a decline and efficient elimination of the test substance and its metabolites from the body at the latest within 72 h post administration. The plasma concentration in the low dose tests as well as the high dose tests was calculated and showed a fast elimination phase after reaching the plasma peak followed by a slower elimination phase after approximately 24 h. There were no sex specific differences in the calculated AUC0 - $_{\infty}$-values for low or high dose male and female rats. Measured dose-normalised kinetic parameters are shown in the table below.

Table B.6.1-6: Measured Dose-Normalised Kinetic Parameters

Dose (mg/kg bw)	**Test 1** **male** **2** single	**Test 2** **female** **2** single	**Test 3** **male** **200** single	**Test 4** **female** **200** single	**Test 5** **male** **2** Repeated dose
Actual dose (mg/kg bw)	1.72	1.93	193.46	221.99	2.34
Compartment model	two	two	two	two	two
t_{max} [h] measured	1	1	2	4	1

t_{max}	[h] calculated	0.92	1.27	3.67	4.49	0.48
C_{max}	[µg/mL] measured	0.493	0.479	0.155	0.122	0.574
C_{max}	[µg/mL] calculated	0.468	0.472	0.179	0.109	0.504
$t_{1/2\ e1}$	[h]	0.18	0.36	2.40	2.96	0.05
$t_{1/2\ e2}$	[h]	44.9	31.5	38.6	88.9	37.8
$AUC_{0-\infty}$	[g/g*h]	3.73	3.63	2.13	2.07	3.22

Metabolism

Isoflucypram was extensively and rapidly metabolized. The majority of metabolites (between 85.61% and 100.79%) were identified (see tables below). No individual metabolite was present, in the urine, in any of the experiments above 10% of the administered dose.

Table B.6.1-7: Metabolites excreted after oral administration of 2 mg/kg bw to male rats expressed as % dose administered

Metabolite (M code *)	**Test 1**		
	(male, 2 mg/kg bw)		
	Urine (0 - 48 h)	Faeces (0 - 48 h)	Total
pyrazole-amide (M64)	0.86	---	0.86
cyclopropyl-pyrazole-carboxamide-OH-GlucA (M61)	0.72	---	0.72
pyrazole-carboxylic acid (M63)	1.59	---	1.59
cyclopropyl-pyrazole-carboxamide-GlucA (isomer 1 and 2) (M60)	2.85	---	2.85
desfluoro-N-methyl-pyrazole-carboxylic acid (M50)	0.50	---	0.50
pyrazole-carboxylic acid-Ala (M65)	4.92	---	4.92
N-methyl-pyrazole-carboxylic acid (M50)	0.67	---	0.67
cyclopropyl-pyrazole-carboxamide (M58)	0.76	---	0.76
cyclopropyl-oxy-pyrazole-carboxamide (M59)	0.31	---	0.31
desmethyl-triOH-GlucA (M34)	---	0.98	0.98
desmethyl-diOH-GlucA (isomer 1) (M31)	---	1.12	1.12
desmethyl-hydroxymethyl-diOH (M08?)	---	2.86	2.86
desmethyl-diOH-GlucA (isomer 3) (M32)	---	0.52	0.52
desmethyl-hydroxyphenyl-1,2-propandiol (M15)	---	8.11	8.11
desmethyl-carboxylicacid-GlucA(isomer 1)and diOH-GlucA (isomer 1 and 2) (M) (M 40 and M 29)	0.17	1.64	1.81
desmethyl-OH-GlucA (isomer 2) (M32)	---	10.24	10.24
desmethyl-hydroxyphenyl-2-propanol (M14)	0.13	4.19	4.32

desmethyl-lactic acid (M09)	---	1.98	1.98
desmethyl-hydroxymethyl-carboxylic acid (M16)	---	3.01	3.01
desmethyl-diOH (isomer) (M08)	---	1.57	1.57
lactic acid (M10)	---	2.67	2.67
propanol-GlucA (isomer 1 and 2) (M19)	---	1.17	1.17
desmethyl-SA (M43)	---	0.28	0.28
desmethyl-carboxylic acid (M11)	---	12.44	12.44
desmethyl-propanol (M06)	---	1.60	1.60
carboxylic acid (M12)	---	12.61	12.61
Propanol (M01)	---	0.95	0.95
2-propanol (M02)	---	0.70	0.70
Desmethyl (M13)	---	1.44	1.44
parent compound	---	4.11	4.11
Total identified	13.46	74.20	87.66
Total characterised	0.54	9.33	9.87
Number of characterised metabolites (maximum value)	11	11	21
Exhaustive extract of faeces	---	2.98	2.98
Solids of faeces (PES)	---	2.58	2.58
Urine or faeces not analysed (48 - 72 h)	0.06	0.20	0.26
Total			103.34

Identified – based on HLPC retention time against a standard
Characterised -unequivocal identification

Table B.6.1-8: Metabolites excreted after oral administration of 2 mg/kg bw to female rats expressed as % dose administered

Metabolite (M Code *)	**Test 2 (female, 2 mg/kg bw)**		
	Urine (0 - 48 h)	Faeces (0 - 48 h)	**Total**
pyrazole-amide (M64)	0.08	---	0.08
cyclopropyl-pyrazole-carboxamide-OH-GlucA (M61)	0.08	---	0.08
pyrazole-carboxylic acid (M63)	0.18	---	0.18
cyclopropyl-pyrazole-carboxamide-GlucA (isomer 1 and 2) (M 60)	0.80	---	0.80
pyrazole-carboxylic acid-Ala (M65)	1.69	---	1.69
N-methyl-pyrazole-carboxylic acid (M50)	0.37	---	0.37
cyclopropyl-pyrazole-carboxamide (M58)	0.60	---	0.60
desmethyl-carboxylicacid-GlucA(isomer 1)and diOH-GlucA (isomer 1 and 2) (M40and M33)	---	1.16	1.16
desmethyl-OH-GlucA (isomer 2) (M32)	0.23	---	0.23
desmethyl-hydroxyphenyl-2-propanol (M14)	1.29	5.45	6.74

desmethyl-hydroxymethyl-carboxylic acid (M16)	0.80	10.58	11.38
desmethyl-carboxylic acid (M11)	4.37	14.47	18.84
desmethyl-propanol (M06)	---	8.68	8.68
carboxylic acid (M12)	0.80	12.98	13.77
Propanol (M01)	---	4.09	4.09
2-propanol (M02)	---	0.64	0.64
Desmethyl (M13)	---	11.43	11.43
parent compound	---	4.86	4.86
Total identified	**11.29**	**74.33**	**85.61**
Total characterised	**0.64**	**9.96**	**10.61**
Number of characterised metabolites (maximum value)	2	8	8
Exhaustive extract of faeces	---	1.86	1.86
Solids of faeces (PES)	---	1.84	1.84
Urine or faeces not analysed (48 - 72 h)	0.07	0.14	0.21
Total			**100.13**

Table B.6.1-9: Metabolites excreted after oral administration of 200 mg/kg bw to male rats expressed as % dose administered

Metabolite (M code *)	**Test 3 (male, 200 mg/kg bw)**		
	Urine (0 - 48 h)	Faeces (0 - 48 h)	**Total**
pyrazole-amide (M64)	0.31	---	0.31
cyclopropyl-pyrazole-carboxamide-OH-GlucA (M61)	0.18	---	0.18
pyrazole-carboxylic acid (M63)	0.16	---	0.16
cyclopropyl-pyrazole-carboxamide-GlucA (isomer 1 and 2) (M60)	0.89	---	0.89
desfluoro-N-methyl-pyrazole-carboxylic acid (M51)	0.19	---	0.19
pyrazole-carboxylic acid-Ala (M63)	3.10	---	3.10
N-methyl-pyrazole-carboxylic acid (M50)	0.30	---	0.30
cyclopropyl-pyrazole-carboxamide (M58)	1.63	---	1.63
desmethyl-hydroxyphenyl-1,2-propandiol (M15)	---	0.76	0.76
desmethyl-OH-GlucA (isomer 2) (M32)	---	2.22	2.22
desmethyl-hydroxyphenyl-2-propanol (M14)	---	3.74	3.74
desmethyl-hydroxymethyl-carboxylic acid (M16)	---	2.31	2.31
desmethyl-diOH (isomer) (M08)	---	0.47	0.47
lactic acid (M10)	---	1.18	1.18
desmethyl-carboxylic acid (M11)	---	10.23	10.23
desmethyl-propanol (M06)	---	1.22	1.22

Metabolite (M code *)	**Test 3 (male, 200 mg/kg bw)**		
	Urine (0 - 48 h)	Faeces (0 - 48 h)	**Total**
carboxylic acid (M12)	---	5.80	5.80
Propanol (M01)	---	0.81	0.81
2-propanol (M02)	---	0.83	0.83
Desmethyl (M13)	---	2.74	2.74
parent compound	---	57.26	57.26
Total identified	**6.76**	**89.58**	**96.34**
Total characterised	**0.32**	**1.13**	**1.45**
Number of characterised metabolites (maximum value)	2	3	5
Solids of faeces (PES)	---	2.59	2.59
Urine or faeces not analysed (48 - 72 h)	0.03	0.14	0.17
Total			**100.55**

Table B.6.1-10: Metabolites excreted after oral administration of 200 mg/kg bw to female rats expressed as % dose administered

Metabolite (M Code)	**Test 4 (female, 200 mg/kg bw)**		
	Urine (0 - 48 h)	Faeces (0 - 48 h)	**Total**
cyclopropyl-pyrazole-carboxamide-OH-GlucA (M61)	0.07	---	0.07
pyrazole-carboxylic acid (M63)	0.16	---	0.16
cyclopropyl-pyrazole-carboxamide-GlucA (isomer 1 and 2) (M60)	0.53	---	0.53
pyrazole-carboxylic acid-Ala (M63)	1.18	---	1.18
N-methyl-pyrazole-carboxylic acid (M50)	0.37	---	0.37
cyclopropyl-pyrazole-carboxamide (M58)	0.40	---	0.40
desmethyl-OH-GlucA (isomer 2) (M33)	---	0.76	0.76
desmethyl-hydroxyphenyl-2-propanol (M14)	0.39	1.64	2.03
desmethyl-hydroxymethyl-carboxylic acid (M16)	0.49	3.63	4.12
lactic acid (M10)	---	0.39	0.39
desmethyl-carboxylic acid (M11)	3.63	4.13	7.76
desmethyl-propanol (M06)	---	3.34	3.34
carboxylic acid (M12)	1.14	5.48	6.62
Propanol (M01)	---	2.79	2.79
2-propanol (M02)	---	0.59	0.59
Desmethyl (M13)	---	5.31	5.31

Metabolite (M Code)	**Test 4 (female, 200 mg/kg bw)**		
	Urine (0 - 48 h)	Faeces (0 - 48 h)	**Total**
parent compound	---	59.07	59.07
Total identified	**8.36**	**87.12**	**95.47**
Total characterised	**0.12**	**2.92**	**3.05**
Number of characterised metabolites (maximum value)	1	4	5
Solids of faeces (PES)	---	1.92	1.92
Urine or faeces not analysed (48 - 72 h)	0.03	0.07	0.10
Total			**100.54**

Table B.6.1-11: Metabolites excreted after repeated oral administration of 2 mg/kg bw for 14 days to male rats expressed as % dose administered

Metabolite (M code)	**Test 5 (male, , 2 mg/kg bw for 14 days)**		
	Urine (0 - 48 h)	Faeces (0 - 48 h)	**Total**
pyrazole-amide (M64)	0.50	---	0.50
cyclopropyl-pyrazole-carboxamide-OH-GlucA (M61)	0.43	---	0.43
pyrazole-carboxylic acid (M63)	1.02	---	1.02
cyclopropyl-pyrazole-carboxamide-GlucA (isomer 1 and 2) (M60)	2.36	---	2.36
desfluoro-N-methyl-pyrazole-carboxylic acid (M51)	0.41	---	0.41
pyrazole-carboxylic acid-Ala (M63)	4.77	---	4.77
N-methyl-pyrazole-carboxylic acid (M53)	0.50	---	0.50
cyclopropyl-pyrazole-carboxamide (M58)	1.67	---	1.67
cyclopropyl-oxy-pyrazole-carboxamide (M59)	0.27	---	0.27
desmethyl-triOH-GlucA (M34)	---	0.54	0.54
desmethyl-hydroxymethyl-diOH (M17)	---	1.55	1.55
desmethyl-diOH-GlucA (isomer 3) (M33)	---	0.31	0.31
desmethyl-hydroxyphenyl-1,2-propandiol (M15)	---	6.07	6.07
desmethyl-carboxylic acid-GlucA (isomer 1) (M40 and M33) and diOH-GlucA (isomer 1 and 2)	---	1.56	1.56
desmethyl-OH-GlucA (isomer 2) (M32)	---	10.47	10.47
desmethyl-hydroxyphenyl-2-propanol (M14)	0.22	3.96	4.18
desmethyl-lactic acid (M09)	---	1.52	1.52
desmethyl-hydroxymethyl-carboxylic acid (M16)	---	6.15	6.15
desmethyl-diOH (isomer) (M08)	---	0.55	0.55
lactic acid (M10)	---	2.20	2.20

propanol-GlucA (isomer 1 and 2) (M19)	---	1.31	1.31
desmethyl-carboxylic acid (M11)	---	13.76	13.76
desmethyl-propanol (M06)	---	4.43	4.43
carboxylic acid (M12)	---	14.34	14.34
Propanol (M01)	---	2.44	2.44
2-propanol (M02)	---	1.19	1.19
Desmethyl (M13)	---	2.98	2.98
parent compound	---	0.73	0.73
Total identified	**12.13**	**76.04**	**88.17**
Total characterised	**0.48**	**9.55**	**10.03**
Number of characterised metabolites (maximum value)	3	10	13)
Exhaustive extract of faeces	---	1.90	1.90
Solids of faeces (PES)	---	2.18	2.18
Urine or faeces not analysed (48 - 72 h)	0.06	0.30	0.36
Fractions not analysed	---	0.04	0.04
Total			**102.68**

Table B.6.1-12: Metabolites excreted after oral administration of 2 mg/kg bw to bile-duct cannulated male rats expressed as % dose administered

Metabolite (M Code *)	**Test 6 (male, bile-duct cannulation, 2 mg/kg bw)**			
	Urine (0 - 24 h)	Bile (0 - 32 h)	Faeces (0 - 24 h)	**Total**
cyclopropyl-pyrazole-carboxamide-OH-GlucA (M61)	0.13	0.15	---	0.28
pyrazole-carboxylic acid (M63)	0.38	0.36	---	0.74
cyclopropyl-pyrazole-carboxamide-GlucA (isomer 1 and 2) (M61)	1.99	1.37	---	3.37
pyrazole-carboxylic acid-Ala (M63)	1.50	0.20	---	1.70
desfluoro-N-methyl-cyclopropyl-pyrazole-carboxamide-OH-Cysanddesfluoro-N-methyl-cyclopropyl-pyrazole-carboxamide-OH-Cys-Gly (M54 and M53)	---	1.57	---	1.57
N-methyl-pyrazole-carboxylic acid (M53)	0.26	---	---	0.26
desfluoro-N-methyl-cyclopropyl-pyrazole-carboxamide-OH-GSH (M54)	---	0.94	---	0.94
cyclopropyl-pyrazole-carboxamide (M58)	0.91	0.09	---	1.00
cyclopropyl-oxy-pyrazole-carboxamide (M59)	0.10	---	---	0.10
desmethyl-triOH-GlucA (M34)	---	2.80	---	2.80
desmethyl-diOH-GlucA (isomer 1) (M29)	---	5.56	---	5.56
desmethyl-diOH-GlucA (isomer 2 and 3) (M33)	---	0.85	---	0.85
desmethyl-hydroxymethyl-diOH (M17)	---	0.36	---	0.36

Metabolite (M Code *)	**Test 6 (male, bile-duct cannulation, 2 mg/kg bw)**			
	Urine (0 - 24 h)	Bile (0 - 32 h)	Faeces (0 - 24 h)	**Total**
desmethyl-diOH-GlucA (isomer 3) (M33)	---	1.52	---	1.52
desmethyl-diOH-GlucA (isomer 4) (M33)	---	2.17	---	2.17
desmethyl-diOH-GlucA (isomer 5) (M33)	---	1.45	---	1.45
desmethyl-OH-GlucA (isomer 1) (M32)	---	1.90	---	1.90
desmethyl-carboxylic acid-GlucA (isomer 1) and diOH-GlucA (isomer 1 and 2) (M31 and M33)	---	1.64	---	1.64
desmethyl-OH-GlucA (isomer 2) (M31)	---	2.09	---	2.09
desmethyl-hydroxyphenyl-2-propanol (M14)	0.10	1.30	0.05	1.44
desmethyl-lactic acid (M09)	---	3.50	---	3.50
desmethyl-hydroxymethyl-carboxylic acid (M16)	---	---	0.20	0.20
desmethyl-diOH (isomer) (M08)	---	---	0.06	0.06
desmethyl-oxo-GlucAcarboxylic acid-GlucA and desmethyl-carboxylic acid-GlucA (isomer 2) (M39, M28 and M 40)	---	4.58	---	4.58
desmethyl-propanol-GlucA(isomer 1)olefine, oxo-GlucA, lacticacid and desmethyl-diOH-GlucA (isomer 6) (M39, MM05, M 30, M10 and M33)	---	13.22	0.02	13.24
propanol-GlucA (isomer 1) (M19)	---	6.98	---	6.98
propanol-GlucA (isomer 2) (M19)	---	3.47	---	3.47
desmethyl-SA (M43)	---	0.19	---	0.19
desmethyl-carboxylic acid (M11)	---	1.51	---	1.51
desmethyl-propanol (M06)	---	0.48	0.11	0.59
carboxylic acid (M12)	---	2.48	0.20	2.68
Propanol (M01)	---	---	0.20	0.20
desmethyl-GlucA (isomer 1) (M35)	---	3.86	---	3.86
desmethyl-GlucA (isomer 2) (M35)	---	1.64	---	1.64
2-propanol (M01)	---	---	0.07	0.07
desmethyl-propanol-GlucA (isomer 2) (M19)	---	0.77	---	0.77
Desmethyl (M13)	---	---	0.38	0.38
parent compound	---	---	18.24	18.24
Total identified	**5.38**	**69.01**	**19.53**	**93.92**
Total characterised	**0.16**	**7.57**	**0.12**	**7.85**
Number of characterised metabolites (maximum value)	1)	19)	1	20)
Solids of faeces (PES)	---	---	0.59	0.59
Urine or faeces (24 - 48 h), bile (32 - 48 h) not analysed	0.35	1.03	0.43	1.81
Fractions not analysed	---	---	0.01	0.01

Metabolite (M Code *)	**Test 6 (male, bile-duct cannulation, 2 mg/kg bw)**			
	Urine (0 - 24 h)	Bile (0 - 32 h)	Faeces (0 - 24 h)	**Total**
Total	**104.16**			
Identification rate of isoflucypram-carboxylic acid (M12) and its metabolites	**5.28**	**9.67**	---	**14.95**

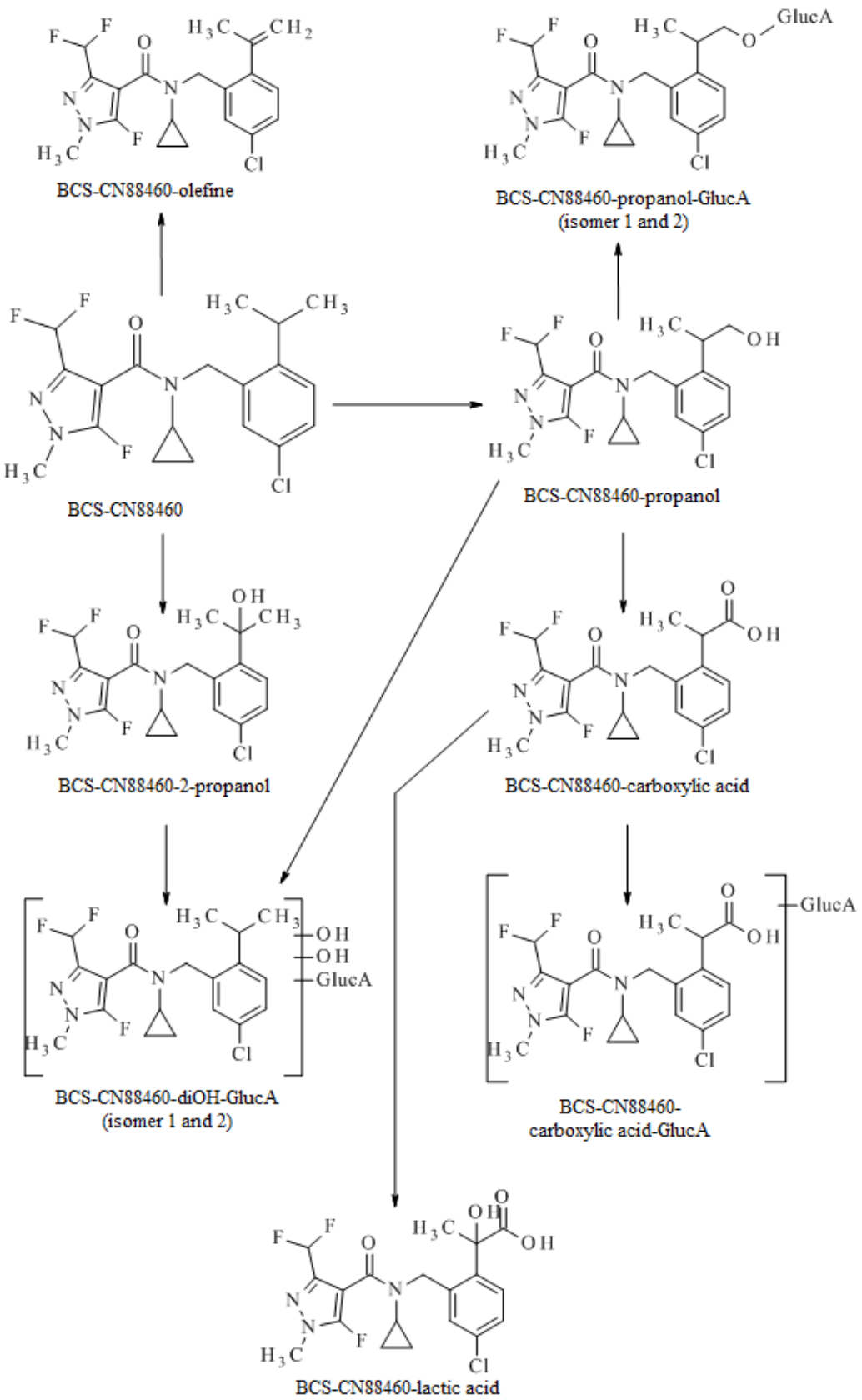
Table B.6.1-13 Metabolites excreted after oral administration of 2 mg/kg bw to bile-duct cannulated female rats expressed as % dose administered

Metabolite (M code*)	**Test 7 (female, bile-duct cannulation, 2 mg/kg bw)**			
	Urine (0 - 48 h)	Bile (0 - 48 h)	Faeces (0 - 48 h)	**Total**
cyclopropyl-pyrazole-carboxamide-GlucA (isomer 1 and 2) (M61)	0.25	---	---	0.25
desfluoro-N-methyl-pyrazole-carboxylic acid (M51)	0.10	---	---	0.10
pyrazole-carboxylic acid-Ala (M65)	0.16	---	---	0.16
desfluoro-N-methyl-cyclopropyl-pyrazole-carboxamide-OH-Cys and desfluoro-N-methyl-cyclopropyl-pyrazole-carboxamide-OH-Cys-Gly (M52 and M53)	---	0.24	---	0.24
N-methyl-pyrazole-carboxylic acid (M30)	0.13	---	---	0.13
desfluoro-N-methyl-cyclopropyl-pyrazole-carboxamide-OH-GSH (M54)	---	0.04	---	0.04
cyclopropyl-pyrazole-carboxamide (M61)	0.13	---	---	0.13
desmethyl-hydroxymethyl-diOH (M17)	---	0.66	---	0.66
desmethyl-diOH-GlucA (isomer 3) (M33)	---	1.42	---	1.42
desmethyl-diOH-GlucA (isomer 4) (M33)	---	2.71	---	2.71
desmethyl-diOH-GlucA (isomer 5) (M33)	---	2.65	---	2.65
desmethyl-OH-GlucA (isomer 1) (M32)	---	0.20	---	0.20
desmethyl-carboxylic acid-GlucA (isomer 1) and diOH-GlucA (isomer 1 and 2) (M40 and M29)	---	2.13	---	2.13
desmethyl-OH-GlucA (isomer 2) (M32)	0.11	3.45	0.10	3.66
desmethyl-hydroxyphenyl-2-propanol (M14)	0.27	1.42	---	1.69
desmethyl-lactic acid (M09)	---	4.36	---	4.36
desmethyl-hydroxymethyl-carboxylic acid (M16)	0.14	---	0.07	0.21
desmethyl-oxo-GlucA carboxylic acid-GlucA* and desmethyl-carboxylic acid-GlucA (isomer 2)* (M39, M28 and M40)	---	4.58	---	4.58
desmethyl-propanol-GlucA (isomer 1), olefine, oxo-GlucA, lactic acid*and desmethyl-diOH-GlucA (isomer 6) (M31, M,05, M30, M10 and M33)	---	11.94	---	11.94
propanol-GlucA (isomer 1) (M19)	---	12.27	---	12.27

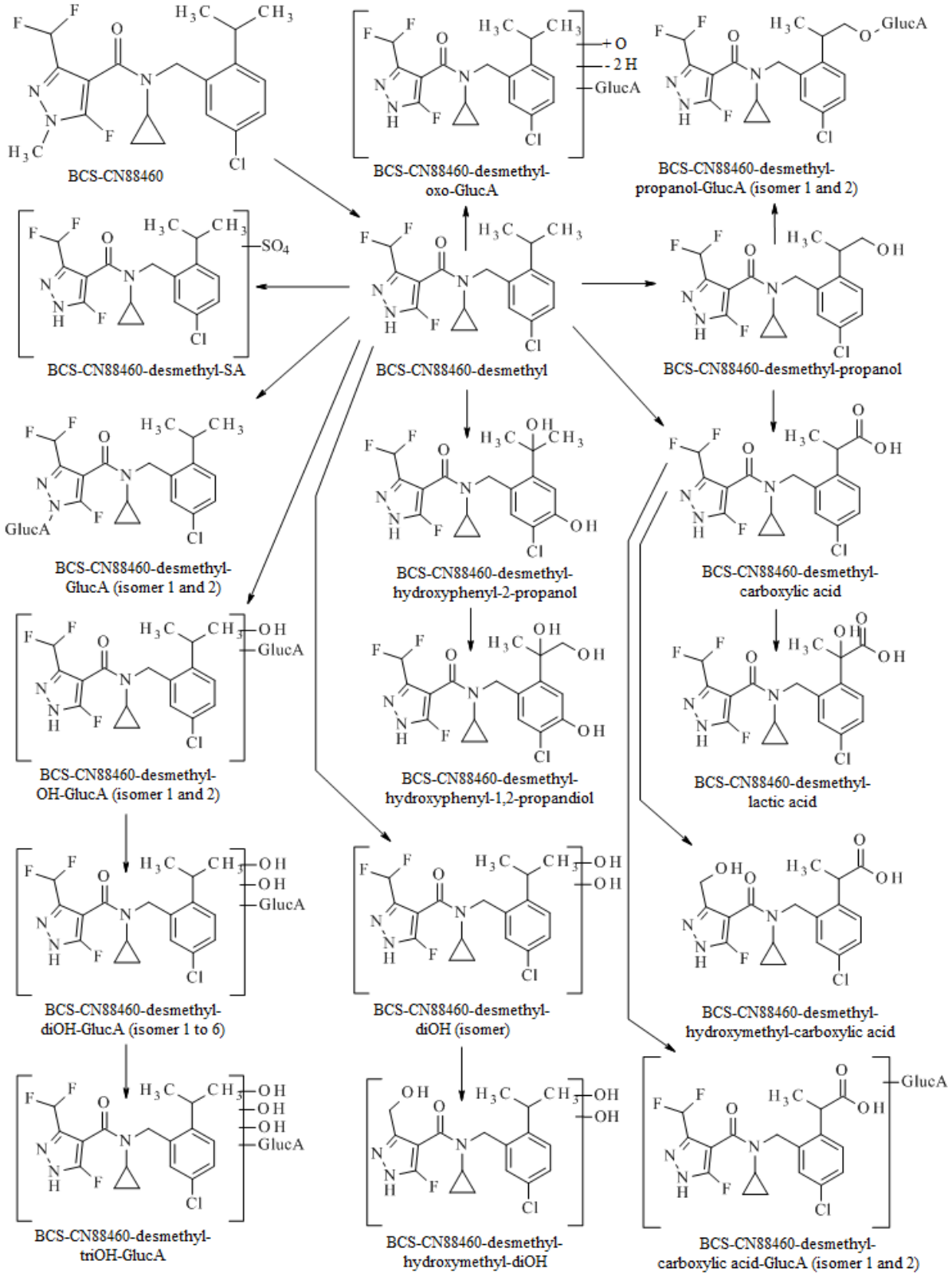
Metabolite (M code*)	**Test 7 (female, bile-duct cannulation, 2 mg/kg bw)**			
	Urine (0 - 48 h)	Bile (0 - 48 h)	Faeces (0 - 48 h)	**Total**
propanol-GlucA (isomer 2) (M19)	---	3.46	---	3.46
desmethyl-SA (M42)	---	0.08	---	0.08
desmethyl-carboxylic acid (M11)	0.87	2.07	---	2.94
desmethyl-propanol (M06)	---	1.15	0.08	1.23
carboxylic acid (M12)	0.13	1.35	0.06	1.54
Propanol (M01)	---	---	0.05	0.05
desmethyl-GlucA (isomer 1) (M35)	---	14.09	---	14.09
desmethyl-GlucA (isomer 2) (M35)	---	7.26	---	7.26
2-propanol (M02)	---	---	0.04	0.04
desmethyl-propanol-GlucA (isomer 2) (M19)	---	4.55	---	4.55
Desmethyl (M13)	---	---	0.46	0.46
parent compound	---	1.24	14.31	15.56
Total identified	**2.30**	**83.33**	**15.17**	**100.79**
Total characterised	**0.07**	**2.12**	---	**2.20**
Number of characterised metabolites (maximum value)	1	8	---	9
Solids of faeces (PES)	---	---	0.35	0.35
Total				**103.34**
Identification rate of isoflucypram-carboxylic acid (M12) and its metabolites	**1.67**	**7.78**	---	**9.46**

Some minor quantitative differences were observed in the metabolic profiles of males and females in the low and high dose tests, however, the RMS does not consider these differences to be toxicologically significant. No relevant qualitative or quantitative differences in the metabolic profile of repeated low-dose male rats were observed in comparison to single dosing. Unchanged parent compound was only detected in the faeces (from 0.73% in the repetated low dose to 59.07% in the single high dose). It is most likely this is a consequence of saturation of absorption from the GIT. The proposed metabolic pathway of isoflucypram is complex and included in the following figures.

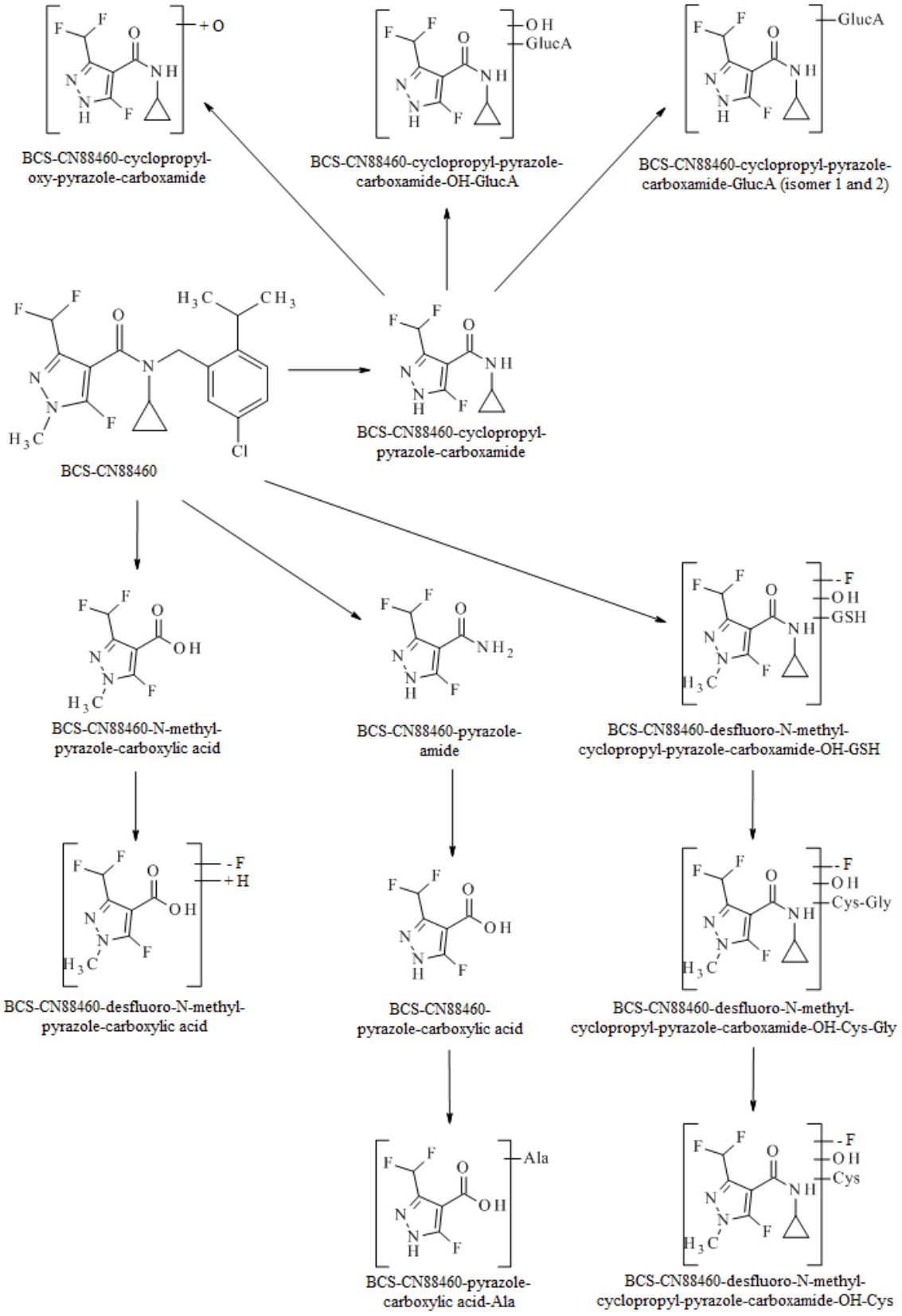
Proposed metabolic pathway of [pyrazole-4-^{14}C] isoflucypram in the rat (Part A)



Proposed metabolic pathway of [pyrazole-4-^{14}C] isoflucypram in the rat (Part B)



Proposed metabolic pathway of [pyrazole-4-^{14}C] isoflucypram in the rat (Part C)



Two prominent Phase I metabolic reactions were identified: N-demethylation of the pyrazole moiety and oxidation of the isopropyl group leading to variety of desmethyl and/or- mono-, di or-tri-hydroxy compounds. The hydroxy

compounds were subject to further oxidative metabolism to desmethyl carboxylate and lactate. The major metabolite in male and female rats was isoflucypram-desmethyl-carboxylic acid (M11) ranging from 7.76% (as sum of urine, bile and faces) of the administered dose in high dose females to 18.9% in low dose females. In bile duct cannulated animals isoflucypram-desmethyl carboxylate only accounted for 3-5% of the administered dose. The prominent Phase II reactions identified were conjugation with amino acids, glucuronic acid and sulphate.

Excretion

Excretion was practically completed 72 h after administration. At this time more than 98% of the recovered dose had been excreted, mainly via the faeces with the remainder in the urine.

In all tests excretion was predominantly via bile and faeces. In bile-duct cannulated rats, approximately 74% of the mean dose recovered was detected in bile samples of male bile-duct cannulated rats and approximately 82% in bile samples of females, respectively.

For single low dose the mean urinary recovery was 12-14 % of the administered dose recovered for males and females. For high dose tests around 7 – 8.4 % of the dose was present in the urine of males and females. The lower urinary excretion rates at the high dose tests indicates that there is a lower absorption of isoflucypram at the higher dose.

Conclusion

The ADME of ^{14}C radiolabelled isoflucypram at the pyrazole ring has been well investigated in rats, in experiments comprising single low (2 mg/kg bw) and high oral dosing (200 mg/kg bw), repeated low dosing and low dose administration in bile duct cannulated animals.

Low dose, ^{14}C pyrazole labelled isoflucypram (2 mg/kg bw) was rapidly and almost completely absorbed from the GIT (absorption - 84-88% of the administered dose in males and females respectively). At the higher dose (200 mg/kg bw) the proportion of unchanged isoflucypram found in the GIT was significantly higher (57-59% of the administered dose) than at the lower dose indicating either reduced absorption and/or increased biliary excretion, both potentially impacting on the systemically available dose. Although uptake across the GIT was high in low dose animals, post hepatic systemic exposure accounted for at least 14.4% of the administered dose, indicating that a significant proportion of isoflucypram absorbed from the GIT is not systemically available. Overall, an **oral absorption** value of **100%** and a **post-hepatic systemic availability value of 15%** have been identified from this study.

Isoflucypram or its metabolites were not found to be retained in any of the investigated tissues and organs. Although blood levels of individual metabolites were not measured, very low levels of total radioactivity were found in terminal blood samples (below 0.1% of the administered dose). ^{14}C pyrazole labelled isoflucypram was extensively and rapidly metabolised; the principal phase I reactions identified were N-demethylation of the pyrazole methyl and/or oxidation of the isopropyl group to desmethyl carboxylates or lactate. The most prominent phase 1 faecal metabolites in low dose males were desmethyl-carboxylic acid or M11 (12.44 of the administered dose) and isoflucypram carboxylic acid or M12 (12.61% of the administered dose). In low dose females, the most prominent faecal metabolites were desmethyl isoflucypram hydroxymethyl-carboxylic acid or M16 (10.38% of the administerd dose in urine, bile and faeces) desmethyl isoflucypram carboxylic acid or M11 (14.47% of the administerd dose in urine, bile and faces) isoflucypram carboxylic acid or M12 (12.98% of the administered dose in urine, bile and faeces) and desmethyl isoflucypram or M13 (11.43% of the administered dose in urine, bile and faeces). The Phase 1 metabolites served as substrates for a number of Phase II reactions, principally glucuronidation. No individual metabolites were detected in the urine at levels approaching 10%. There were no pronounced sex differences in metabolite profiles or between low and high dose rats. There was almost no systemic exposure to unchanged parent.

Excretion of most isoflucypram metabolites was via the bile, as glucuronides and other conjugates. Investigations in bile duct cannulated rats found that biliary excterion of the single low dose of isoflucypram is rapid and essentially complete after 24 hours. There is some evidence of enterohepatic recirculation as the amount recovered in the urine, in low dose non bile duct cannulated animals is around 8-10% higher than in bile-duct cannulated animals. Urinary excretion accounted for a maximum of 12-14% of the administered dose, in low dose male and female rats.

([redacted] *2017a)*

Study 2: ADME – ^{14}C phenyl isoflucypram

Study	Amendment no 1: Phenyl-UL-14C]BCS-CN88460-Absorption, Distribution, Excretion and Metabolism in the Rat
Reference	[redacted] (2017b)
Date performed	27/09/2017
Test facility	[redacted]
Report reference	M1824611-3
Guideline(s)	OECD 417
Deviations from the guideline	None
GLP	Yes
Test material	^{14}C phenyl – 99.9% (radiochemical purity >99.9%)
Study acceptable	Yes

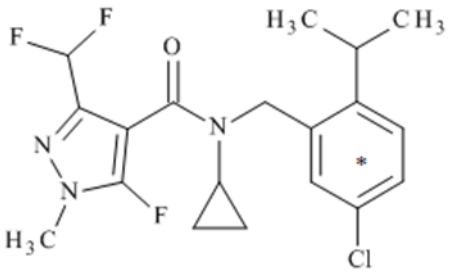
Methods

The toxicokinetis of isoflucypram, labelled with ^{14}C in the phenyl moiety, was investigated in male and female Wistar rats. Two experiments with male and female rats at a low dose of 2 mg/kg bw were conducted in this study. The rats received the radiolabelled isoflucypram by oral gavage as a suspension in water and tragacanth at pH 4. They were sacrificed three days post dosing. The total radioactivity included the radioactivity related to the test compound and the metabolites and was determined in plasma, urine, faeces, organs and tissue samples at sacrifice. Metabolites were investigated in urine and extracts of faeces. In this study, TOPFIT version 2.0 was used to calculate toxicokinetic parameters by plasma concentration-time curve analysis and a standard 2-compartment disposition model was applied for curve fitting computation. Further details of the experimental design are included in table B.6.6-14 below.

Table B.6.1-14: Study design

Experiment	**Numbers/Dose/Dose regimen**	**Time of sample collection**			
		Urine [h]	**Faeces** [h]	**Organs** [h]	**Microplasma** [h]
1	4 male rats single oral dose (2 mg/kg bw)	4, 8, 12, 24, 48, 72	24, 48, 72	72	0.25, 0.5, 1, 2, 4, 7, 24, 48, 72
2	4 female rats single oral dose (2 mg/kg bw)	4, 8, 24, 48, 72	24, 48, 72	72	

Figure B.6.1-2: Isoflucypram showing position of radiolabel



Results

Recovery was good, with 102-104% of the administered dose accounted for (see table B.6.1-15 below).

Table B.6.1-15: Recovery of radioactivity in excreta, gastrointestinal tract and the body of rats following oral dosing of [phenyl-UL-^{14}C]isoflucypram

	Percent of total radioactive dose administered (mean values)		**Percent of total radioactive dose recovered (mean values)**	
	Test 1 male oral 2 mg/kg bw	**Test 2** female oral 2 mg/kg bw	**Test 1** male oral 2 mg/kg bw	**Test 2** female oral 2 mg/kg bw
Urine	10.89	8.39	10.66	7.99
Faeces	91.04	96.21	88.98	91.75
Total excreted	**101.93**	**104.59**	**99.64**	**99.74**
Body excluding GIT	0.290	0.234	0.283	0.223
GIT	0.077	0.036	0.076	0.034
Total in body	**0.367**	**0.270**	**0.359**	**0.257**
Balance	**102.29**	**104.86**	**---**	**---**
Norm. - factor	---	---	0.978	0.954

Absorption

The absorption of phenyl-^{14}C isoflucypram started immediately after oral administration, as indicated by the presence of radioactivity in plasma samples after 15 minutes. For both experiments, the maximum plasma concentration (Cmax) was reached at 1 h (tmax) after administration with a mean concentration of 0.417 mg active substance equivalent / kg $_{sample}$ in male rats and 0.498 mg active substance equivalent / kg $_{sample}$ in female rats, respectively. The time course of the mean plasma levels was comparable in male and female rats. A quantitative estimate of absorption was not specifically determined in this study; it is unclear why this was not part of the study protocol.

Distribution

The equivalent concentration of radioactivity in organs and tissues of male and female rats was in the same order of magnitude (see table below). At sacrifice low levels (mean values: 0.290% in males and 0.234% in females of the administered radioactivity) were found in the carcass excluding GIT. Low amounts of the administered dose were detected in the GITs (0.077% in males and 0.036% in females) at the end of the study.

The highest concentration of radioactivity was detected in the liver with mean equivalent concentrations of 0.0347 mg/kg liver in males and 0.0374 mg/kg liver in female rats. When compared to other organs, the concentration in blood cells was around 2-20-fold higher, but still low in absolute terms.

Mean equivalent concentrations were 0.0225 mg/kg in blood cells of male rats and 0.0295 mg/kg in blood cells of female rats. The mean equivalent concentration in the other organs and tissues ranged from 0.0011 mg/kg to 0.0115 mg/kg.

Table B.6.1-16: Radioactive residues in organs and tissues at sacrifice (72 hours) after oral administration of [phenyl-UL-^{14}C] isoflucypram

	Equivalent concentration [mg active substance equivalent / kg sample] (mean values)		**Dose normalised concentration (mean values)**	
Organs/ Tissues	Test 1 oral male 2 mg/kg bw	Test 2 oral female 2 mg/kg bw	Test 1 oral male 2 mg/kg bw	Test 2 oral female 2 mg/kg bw
Blood Cells	0.0225	0.0295	0.0134	0.0156
Plasma	0.0074	0.0071	0.0044	0.0038
Carcass	0.0035	0.0029	0.0021	0.0015
Heart	0.0069	0.0057	0.0041	0.0030
Brain	0.0016	0.0011	0.0010	0.0006
Kidneys	0.0115	0.0090	0.0069	0.0047
Liver	0.0347	0.0374	0.0207	0.0197
Testes	0.0019	---	0.0011	---
Ovaries	---	0.0046	---	0.0025
Uterus	---	0.0049	---	0.0026
Adrenal gland	0.0078	0.0057	0.0047	0.0030
Harderian gland	0.0048	0.0039	0.0029	0.0021
Thyroid gland	n.c.	n.c.	n.c.	n.c.
Spleen	0.0053	0.0099	0.0032	0.0052
Lung	0.0083	0.0096	0.0050	0.0050
Eyes	0.0014	0.0013	0.0009	0.0007
Skin	0.0033	0.0038	0.0019	0.0020
Bone femur	0.0024	n.c.	0.0014	n.c.
Perirenal fat	0.0113	0.0101	0.0068	0.0053
Muscle leg	0.0021	0.0017	0.0013	0.0009

For both experiments, the mean concentration of the total radioactivity in plasma declined to values below 1.93% of the maximum concentration within 72 h post administration. This indicates no significant retention of compound related residues in the body of the animals. The plasma concentration was calculated with a two-compartment model by TOPFIT. There were no relevant sex specific differences in the measured or calculated pharmacokinetic parameters. Toxicokinetic parameters determined in this study are presented in table B.6.1-17 below.

Table B.6.1-17 toxicokinetic parameters

Dose (mg/kg bw)	**Test 1** male 2 oral	**Test 2** female 2 oral
Actual dose (mg/kg bw)	1.63	1.88
Compartment model	two	two
t_{max} [h] measured	1	1
t_{max} [h] calculated	0.72	0.83
C_{max} [µg/mL] measured	0.255	0.265
C_{max} [µg/mL] calculated	0.248	0.222

Dose (mg/kg bw)	**Test 1** male 2 oral	**Test 2** female 2 oral
$t_{1/2\ a}$ [h]	0.10	0.11
$t_{1/2\ e}$ [h]	44.5	31.1
$AUC_{0-\infty}$ [g/g*h]	2.43	2.75

Metabolism

Isoflucypram was extensively and rapidly metabolized. The majority of metabolites were quantified (see tables B.6.1-18 and B.6.1-19 below). In males, the most abundant faecal metabolites were: desmethyl-hydroxyphenyl-1,2-propandiol or M15 (11.41% of the administered dose) and desmethyl-diOH or M08 (14.6%) in males. In females the most abundant faecal metabolites were: desmethyl-diOH or M08 (19.35% of the administered dose) desmethyl-carboxylic acid or M11 (11.09% of the administered dose), desmethyl-propanol or M06 (14.24% of the administered dose) and isoflucypram carboxylic acid or M12 (14.16% of the administered dose). No individual urinary metabolite exceeded 10% of the administered dose.

Table B.6.1-18: Metabolites excreted after oral administration of 2 mg/kg bw to male rats

Metabolite (M codes)	**Test (male, 2 mg/kg bw)**		
	Urine (0 – 48 h)	Faeces (0 – 48 h)	**Total**
	% of dose administered		
benzylalcohol-dioxo-GlucA (isomer 1) (M75)	0.82	---	0.82
benzylalcohol-dioxo-GlucA (isomer 2) (M75)	1.99	---	1.99
desmethyl-triOH-GlucA (M34)	---	0.84	0.84
benzylalcohol-dioxo (isomer 1) (M74)	0.38	---	0.38
desmethyl-hydroxymethyl-diOH (M17)	---	3.31	3.31
benzylalcohol-dioxo (isomer 2) (M74)	0.55	---	0.55
desmethyl-hydroxyphenyl-1,2-propandiol (M15)	---	11.41	11.41
desmethyl-carboxylic acid-GlucA (isomer 1) and diOH-GlucA (isomer 1 and 2) (M40 and M29)	---	4.38	4.38
phenyl-formyl-olefine, benzylalcohol-GlucA and benzylalcohol-oxo-GlucA (isomer 1) (M76, M70 and MM73)	1.45	---	1.45
desmethyl-diOH (group of isomers) (M08)	---	14.60	14.50
benzylalcohol-oxo-GlucA (isomer 2) (M73)	1.64	---	1.64
desmethyl-lactic acid (M09)	---	6.53	6.53
desmethyl-diOH (isomer) (M08)	---	7.41	7.41
lactic acid (M10)	---	3.58	3.58
propanol-GlucA (isomer 1 and 2) (M19)	---	1.10	1.10
benzylalcohol-oxo-desdihydro (isomer 1) (M72)	0.14	---	0.14
benzylalcohol-oxo desdihydro (isomer 2) (M72)	0.11	---	0.11

Metabolite (M codes)	Test (male, 2 mg/kg bw)		
	Urine (0 – 48 h)	Faeces (0 – 48 h)	**Total**
	% of dose administered		
desmethyl-SA (M43)	---	0.41	0.41
desmethyl-carboxylic acid (M11)	---	7.48	7.48
desmethyl-propanol (M06)	---	2.63	2.63
carboxylic acid (M12)	---	9.76	9.76
Propanol (M1)	---	4.04	4.04
benzylalcohol-oxo (M7)	1.13	---	1.13
2-propanol (M02)	---	1.12	1.12
parent compound	---	1.08	1.08
Total identified	**8.21**	**79.68**	**87.89**
Total characterised	**2.51**	**6.84**	**9.35**
Number of characterised metabolites (maximum value)	11	6	17
Exhaustive extract of faeces	---	2.76	2.76
Solids of faeces (PES)	---	1.50	1.50
Urine or faeces not analysed (48 - 72 h)	0.17	0.25	0.42
Total			**101.93**

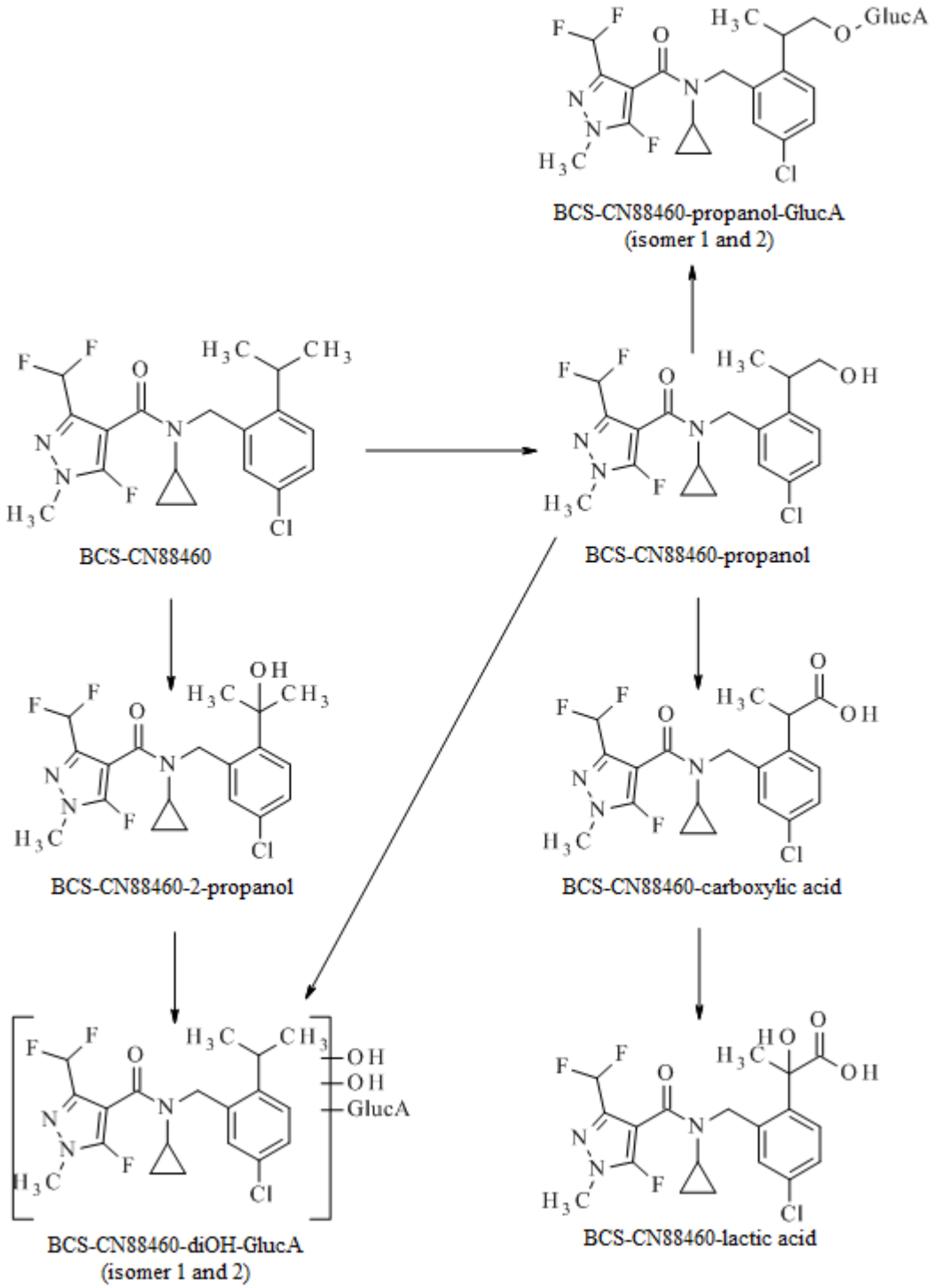
Table B.6.1-19 Metabolites excreted after oral administration of 2 mg/kg bw to female rats

Metabolite (M code)	Test 2 (female, 2 mg/kg bw)		
	Urine (0 – 48 h)	Faeces (0 – 48 h)	**Total**
	% of dose administered		
benzylalcohol-dioxo-GlucA (isomer 1) (M75)	0.15	---	0.15
benzylalcohol-dioxo-GlucA (isomer 2) (M73)	0.54	---	0.54
benzylalcohol-dioxo (isomer 1) (M74)	0.21	---	0.21
benzylalcohol-dioxo (isomer 2) (M74)	0.38	---	0.38
desmethyl-carboxylic acid-GlucA (isomer 1) and diOH-GlucA (isomer 1 and 2) (M40 and M29)	---	2.50	2.50
phenyl-formyl-olefine, benzylalcohol-GlucA and benzylalcohol-oxo-GlucA (isomer 1) (M76, M70 and MM73)	1.45	---	1.45
desmethyl-diOH (group of isomers) (M08)	---	1.13	1.13

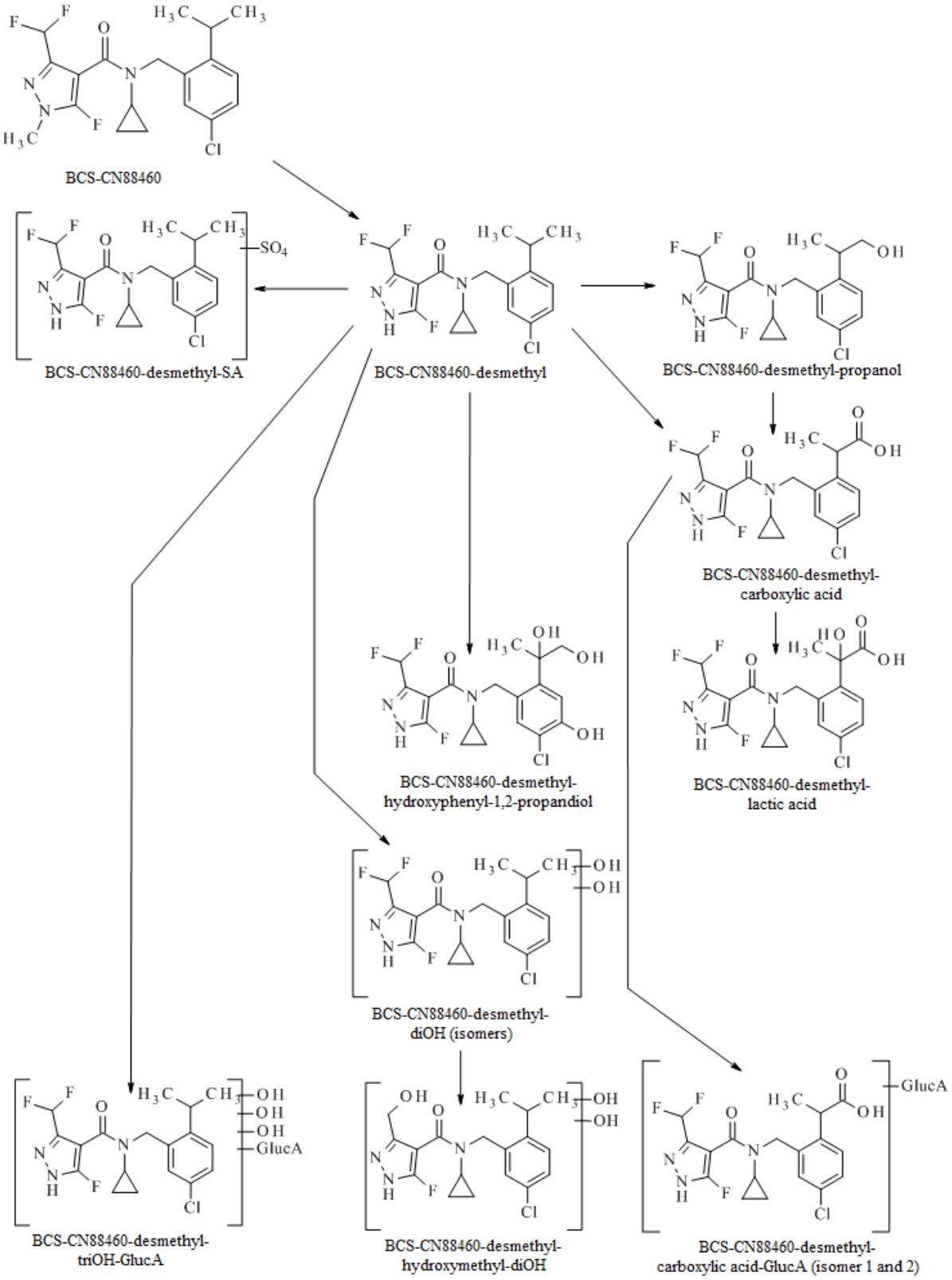
Metabolite (M code)	Test 2 (female, 2 mg/kg bw)		
	Urine (0 – 48 h)	Faeces (0 – 48 h)	**Total**
	% of dose administered		
benzylalcohol-oxo-GlucA (isomer 2) (M73)	0.78	---	0.78
desmethyl-lactic acid (M09)	---	4.06	4.06
desmethyl-diOH (isomer) (M08)	---	19.35	19.35
lactic acid (M10)	---	0.28	0.28
propanol-GlucA (isomer 1 and 2) (M19)	---	1.13	1.13
benzylalcohol-oxo-desdihydro (isomer 1) (M72)	0.11	---	0.11
benzylalcohol-oxo desdihydro (isomer 2) (M72)	0.26	---	0.26
desmethyl-SA (M43)	---	1.40	1.40
desmethyl-carboxylic acid (M11)	1.31	11.09	12.40
desmethyl-propanol (M06)	---	14.24	14.24
carboxylic acid (M12)	0.39	14.16	14.55
Propanol (M1)	---	8.92	8.92
benzylalcohol-oxo (M7)	0.45	---	0.45
2-propanol (M02)	---	0.88	0.88
Desmethyl (M13)	---	2.59	2.59
parent compound	---	4.12	4.12
Total identified	**6.03**	**85.83**	**91.86**
Total characterised	**2.16**	**5.13**	**7.29**
Number of characterised metabolites (maximum value)	8	5	13
Exhaustive extract of faeces	---	3.15	3.15
Solids of faeces (PES)	---	1.71	1.71
Urine or faeces not analysed (48 - 72 h)	0.20	0.39	0.59
Total			**104.59**

The important metabolic reactions of [phenyl ^{14}C] isoflucypram were N-demethylation of the pyrazole moiety (>50%), oxidation of the isopropyl group resulting in the formation of carboxylic and lactic acids, and Phase II conjugates of these. In this study, a number of phenyl-derived metabolites (benzylalcohol compounds and related metabolites), which resulted from cleavage of the pyrazole moiety, were identified. These metabolites were not identified in the previous study, due to the position of the radiolabel (in the pyrazole ring). Metabolites specific for the pyrazole-label were identified in ADME study 1 ([redacted] (2017a). The most prominent Phase II reaction was glucuronidation, accounting for around 11% of the administered dose and was more pronounced in males. The proposed metabolic pathways are detailed over the page.

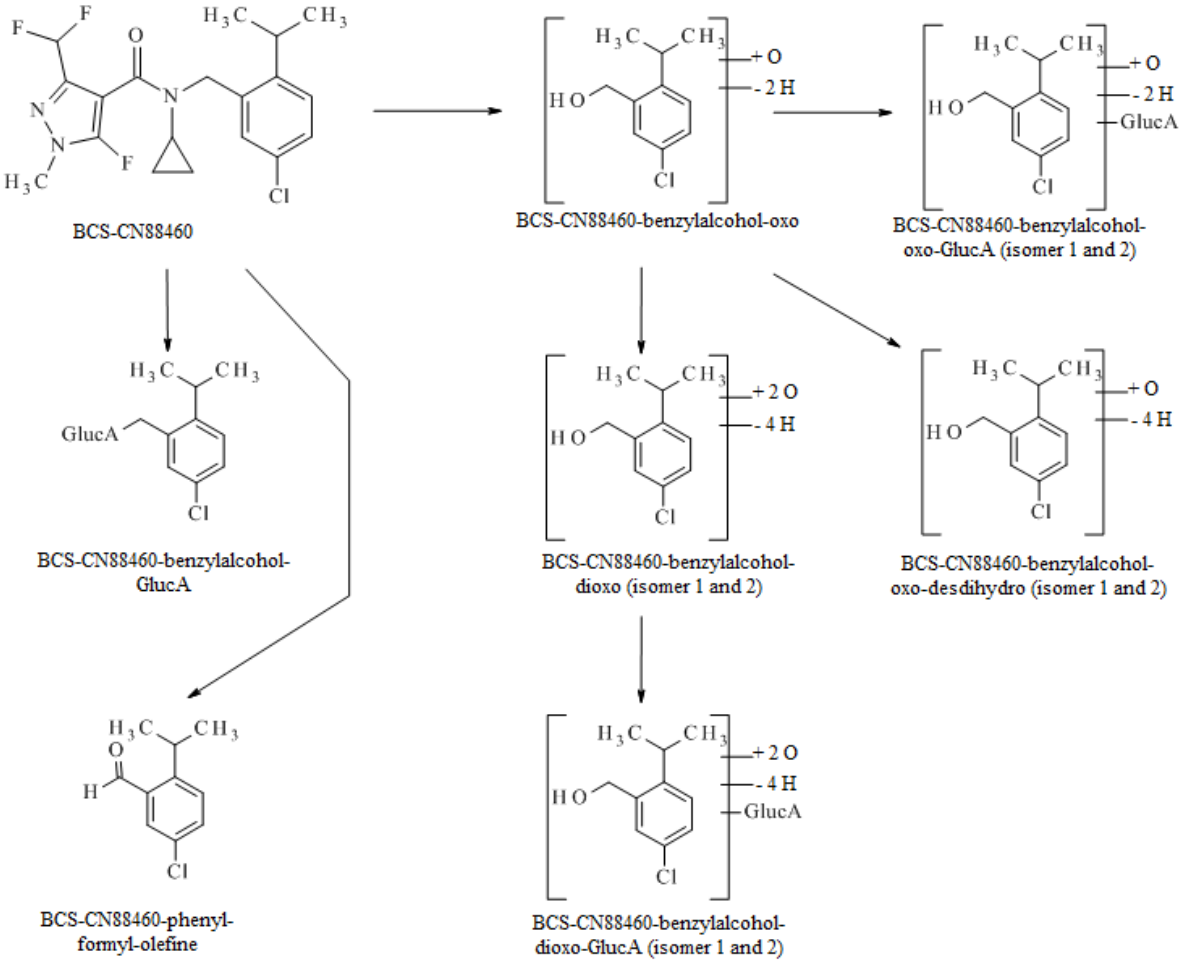
Proposed metabolic pathway of [phenyl-UL-^{14}C]isoflucypram in the rat (Part A)



Proposed metabolic pathway of [phenyl-UL-^{14}C]Isoflucypram in the rat (Part B)



Proposed metabolic pathway of [phenyl-UL-^{14}C]isoflucypram in the rat (Part C)



Excretion

The excretion in male and female rats was almost completed 72 h after administration. At this time more than 99% of the recovered dose had been excreted via urine and faeces. In both tests the main portion of radioactivity (>80%) was excreted within 24h. In both sexes, excretion was predominantly faecal and amounted around 90% of the recovered radioactivity. Urinary excretion (mean values) accounted for 11% and 8% of the recovered radioactivity for males and females respectively.

Conclusion

Overall, low dose ^{14}C phenyl labelled isoflucypram (2 mg/kg bw) was rapidly absorbed after oral gavage dosing to rats. Isoflucypram or its metabolites were not found to be retained in any of the investigated tissues and organs. Although blood levels of individual metabolites were not measured, very low levels of total radioactivity were found in terminal blood samples (below 0.1% of the recovered radioactivity). ^{14}C pyrazole labelled isoflucypram was metabolized via N-demethylation of the pyrazole methyl and/or oxidation of the isopropyl group to desmethyl carboxylates or lactate. A number of metabolites were identified in this study, resulting from cleavage of the pyrazole moiety leading to a number of benzylalcohol compounds and related metabolites. The most prominent Phase II reaction was glucuronidation, accounting for around 11% of the administered dose and was more pronounced in males.

There was almost no systemic exposure to unchanged parent. No prominent sex differences in metabolism were observed. Excretion of most metabolites was via the faeces, as glucuronides and other conjugates, with around 90% of the recovered radioactivity eliminated via this route. Urinary excretion accounted for 8-11% of the total administered dose, with no individual urinary metabolite present above 10%.

([redacted] *2017b)*

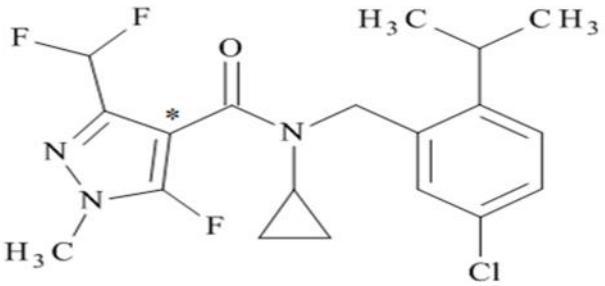
Study 3: Quantitative Whole-Body Autoradiography – ^{14}C pyrazole labelled isoflucypram

Study	[Pyrazole-4-14C]BCS-CN88460: Distribution of the total radioactivity in male and female rats determined by quantitative whole body autoradiography, determination of the exhaled $^{14}CO2$, and pilot metabolism experiments
Reference	[redacted] (2017c)
Date performed	21/09/17
Test facility	[redacted]
Report reference	M1812170-0
Guideline(s)	OECD 417
Deviations from the guideline	None
GLP	Yes
Test material	^{14}C pyrazole – 99% (radiochemical purity >99%)
Study acceptable	Yes

Methods

The toxicokinetic behaviour of ^{14}C pyrazole labelled isoflucypram was investigated in male and female Wistar rats using a single oral gavage dose of 5 mg/kg bw. The absorption of radioactivity from the GI-tract, distribution to and elimination from blood, organs, and tissues were analysed qualitatively and quantitatively by whole body autoradioluminography (WBAL). For both sexes, one rat each was taken for cryosectioning at 1, 4, 8, 24, 48, 72, 120, and 168 h after administration (Test 2: male; Test 3: female). The amounts of radioactivity in excreta (urine and faeces) and exhaled carbon dioxide were determined for selected time periods. Limited investigations of metabolism were conducted using single male rats, sacrificed at 1, 4, 8, and 24 h after administration (Test 1). Samples of urine, faeces, plasma, liver, kidney, and thyroid were collected and afterwards prepared for the chromatographic evaluation of the metabolic profiles by HPLC with radiometric detection. The recoveries of radioactivity in this animal are reported as a percentage of the total recovery from the organ/tissue, not total radioactivity recovered from the whole animal.

Figure B.6.1-3: ^{14}C pyrazole isoflucypram



Results

In these two experiments, recoveries were around 88.63-100% in females and males, respectively.

Absorption

The radiolabel was absorbed very rapidly from the gastrointestinal tract of male and female Wistar rats after single oral administration leading to maximum plasma levels 1-h after dosing. The extent of absorption or systemic availability was not specifically determined in this study.

Distribution

Radioactivity was widely distributed immediately post-dosing in both males and females, most abundantly in the liver and kidney. Although minimal in absolute terms, maximum concentrations of radioactivity were reached 1 h after dosing in all organs and tissues in both sexes. At terminal sacrifice (168 h), trace amounts of radioactivity were detected only in blood, liver, kidney, lung, adrenal gland, and nasal mucosa of male rats, as well as in blood, liver, and nasal mucosa of female rats. No sex differences were observed in distribution of common organs and tissues. It is notable that the bone marrow was exposed from 1-24-hours post dosing.

Metabolism

In the metabolism investigations in the single dose male rat, no unchanged parent was detected in plasma samples. After 1-hour the most abundant metabolites in plasma, were cyclopropyl-pyrazole-carboxamide or M61 (14.4% of total recovered radioactivity from the plasma), isoflucypram desmethyl carboxylic acid or M11 (34.3% of total recovered radioactivity from the plasma) and isoflucypram desmethyl lactic acid or M10 (5.4% of recovered radioactivity from the plasma).

In liver samples up to 35 metabolites were detected beside the parent compound. The most abundant metabolites identified and quantified and at1 h after administration were: isoflucypram-carboxylic acid or M12 (21.9% of total recovered radioactivity from the liver) desmethyl isoflucypram carboxylic acid or M11 (14.8% of total recovered radioactivity from the liver) and isoflucypram lactic acid or M10 (7.6% of total recovered radioactivity from the liver at 1 hr).

Unchanged parent compound could not be detected in urine. Three prominent urinary metabolites identified were: pyrazole-carboxylic acid or M63 (0.33% of administered dose 0-24h), cyclopropyl-pyrazole-carboxamide-GlucA (isomer 1) or M60 (3.29% of administered dose 0-24h), and cyclopropyl-pyrazole-carboxamide or M61 (0.54% of administered dose 0-24h). In comparison, unchanged isoflucypram was the largest fraction that was detected in the faeces extract (13.17% of dose). The most abundant faecal metabolites (0-24h) were: desmethyl-carboxylic acid or M11 (12.56% of the administered dose), isoflucypram carboxylic acid or M12 (8.89% of the administered dose), desmethyl-hydroxyphenyl-1,2-propandiol or M15 (6.75% of the administered dose) and desmethyl-hydroxyphenyl-2-propanol or M14 (6.38% of the administered dose). These faecal metabolite findings compare well with the earlier toxicokinetic study in which rats were administered a single oral dose of 2 mg/kg bw isoflucopyram ([redacted] 2017a).

The most important metabolic reactions of pyrazole ^{14}C labelled isoflucypram were demethylation of the pyrazole moiety (>50%), oxidation of the isopropyl group resulting in the formation of carboxylic and lactic acids and Phase II conjugates of these. The most prominent Phase II reaction was glucuronidation.

Excretion

In both males and females 69-91% of the total radioactivity was excreted within 24 h after dosing, almost exclusively via the faeces (see tables B.6.1-20 and B.6.1-21 below).

Table B.6.1-20: Cumulative excretion of radioactivity in urine, faeces and expired air of male rats after a single oral administration of 5 mg [pyrazole-4-^{14}C] isoflucypram /kg bw

	Percent of radioactive dose administered (cumulative)							
	Animal no.							
	268	269	270	271	272	273	274	275
	Time of sacrifice [h after administration]							
	1	**4**	**8**	**24**	**48**	**72**	**120**	**168**
Exhaled air								

	Percent of radioactive dose administered (cumulative)							
	Animal no.							
	268	269	270	271	272	273	274	275
	Time of sacrifice [h after administration]							
	1	**4**	**8**	**24**	**48**	**72**	**120**	**168**
24 h					0.0050	0.0069	0.0050	0.0046
48 h					0.0059	0.0079	0.0056	0.0053
Urine								
1 h	0.04							
4 h		3.44	2.27	3.18	1.57	3.65	4.39	1.32
8 h			6.23	5.04	2.63	7.39	8.15	6.55
24 h				9.22	6.29	12.32	13.21	11.39
48 h					6.62	12.91	13.56	11.74
72 h						12.99	13.60	11.82
96 h							13.64	11.86
120 h							13.66	11.90
144 h								11.92
168 h								11.93
Faeces								
24 h	*	*	*	82.21	72.83	74.86	75.04	80.02
48 h					85.62	89.73	83.44	87.58
72 h						90.86	84.21	88.06
96 h							84.30	88.15
120 h							84.35	88.20
144 h								88.24
168 h								88.27
Sum total	**0.04**	**3.44**	**6.23**	**91.43**	**92.25**	**103.86**	**98.02**	**100.20**

* Non detected

Table B.6.1-21: Cumulative excretion of radioactivity in urine, faeces and expired air of female rats after a single oral administration of 5 mg [pyrazole-4-^{14}C] isoflucypram /kg bw

	Percent of radioactive dose administered (cumulative)							
	Animal no.							
	276	277	278	279	280	281	282	283
	Time of sacrifice [h post administration]							
	1 h	**4 h**	**8 h**	**24 h**	**48 h**	**72 h**	**120 h**	**168 h**
Exhaled air								
24 h					0.0020	0.0011	0.0014	0.0016
48 h					0.0024	0.0012	0.0015	0.0019
Urine								
1 h	0.07							
4 h		3.32	0.07	0.53	2.98	1.50	0.27	0.25
8 h			2.68	2.02	3.26	1.96	2.69	2.38
24 h				6.80	11.49	7.10	6.89	6.98
48 h					13.66	7.71	7.84	8.20

	Percent of radioactive dose administered (cumulative)							
	Animal no.							
	276	277	278	279	280	281	282	283
	Time of sacrifice [h post administration]							
	1 h	**4 h**	**8 h**	**24 h**	**48 h**	**72 h**	**120 h**	**168 h**
72 h						7.84	7.94	8.31
96 h							7.96	8.33
120 h							7.98	8.34
144 h								8.35
168 h								8.35
Faeces								
24 h	*	*	*	61.83	47.50	67.59	56.16	60.24
48 h					74.27	79.03	75.44	78.69
72 h						80.22	76.63	80.01
96 h							76.77	80.18
120 h							76.80	80.20
144 h								80.25
168 h								80.28
Sum total	**0.07**	**3.32**	**2.68**	**68.63**	**87.94**	**88.06**	**84.78**	**88.63**

* Non detected

Conclusion

^{14}C phenyl labelled isoflucypram (5 mg/kg bw) was rapidly absorbed after oral gavage dosing to rats. Isoflucypram, or its metabolites were not found to be retained to any significant extent in any of the investigated tissues and organs.

Low levels of radioactivity were found in initial blood samples which declined rapidly post dosing. There was almost no systemic exposure to unchanged parent. ^{14}C pyrazole labelled isoflucypram was rapidly and extensively metabolized in the liver via N-demethylation of the pyrazole methyl and/or oxidation of the isopropyl group to desmethyl carboxylates or lactate. The most prominent Phase II reaction was glucuronidation. Investigation of plasma metabolites found the most abundant metabolite was the desmethyl carboxylic acid. This metabolite was not detected in urine samples. No prominent sex differences in metabolism were observed. Excretion of most metabolites primarily as glucuronides, presumably was via the bile. Urinary excretion accounted 8-14% of the total administered dose, with no individual metabolite present above 10%. No radiolabel was detected in the expired air. Overall, WBAL demonstrated that male and female rats exhibit a very similar absorption, distribution, and excretion with no potential for retention of either unchanged parent, or metabolites.

([redacted] *2017c)*

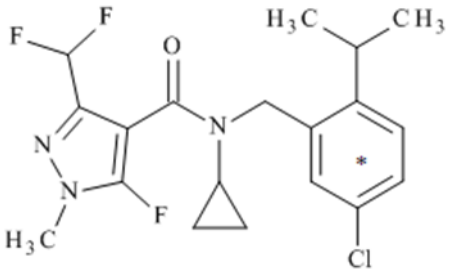
Study 4: Quantitative Whole-Body Autoradiography - ^{14}C phenyl isoflucypram

Study	Amendment 1 to [phenyl-UL-^{14}C]BCS-CN88460: Tissue Distribution and Excretion ofRadioactivity in the Rat by Quantitative Whole Body Autoradiography – Amended final report 1
Reference	[redacted] (2017)
Date performed	19/05/17
Test facility	[redacted]
Report reference	[redacted] Study Number - 8342252
Guideline(s)	OECD 417
Deviations from the guideline	None
GLP	Yes
Test material	^{14}C pyrazole – 99% (radiochemical purity >99%)
Study acceptable	Yes

Methods

In this study the tissue distribution and toxicokinetic parameters of ^{14}C phenyl labelled isoflucypram was investigated in male and female rats (Wistar strain) following a single oral administration at a dose of 5 mg/kg bw. Following dosing, animals were housed singly in metabowles. Blood samples were taken at 15 minutes, 1, 2, 7, 24, 48, 72, 96, 120, 144 and 168 hours post-dose for toxicokinetic analysis. Single animals were sacrificed at 1, 4, 8, 24, 48, 72, 120 and 168 hours post-dose for analysis of tissue distribution using qquantitative whole-body autoradiography. A full mass-balance was not established as carcasses were retained for quantitative whole body autoradiography.

Figure B.6.1-4: Isoflucypram – showing position of radiolabel (* uniform)



Results

Radioactivity was widely distributed immediately post-dosing in both males and females, most abundantly in the liver kidney, bile ducts. Although minimal in absolute terms, maximum concentrations of radioactivity were reached 1 h after dosing in all organs and tissues in both sexes. At terminal sacrifice (168 h), radioactivity was not quantifiable in any tissue. No sex differences were observed in distribution of common organs and tissues. It is notable that the bone marrow was exposed from 1-24-hours post dosing.

The concentrations of [phenyl-UL-^{14}C] isoflucypram in terms of µg equivalents/g and pharmacokinetic parameters (Cmax, $T_{1/2}$ and AUC) were determined. Peak concentrations (Cmax) in plasma of 1.038 and 0.800 µg equiv/g were measured at 1 and 2 hours in males and females, respectively. The elimination half-life was 18.4 hours in male plasma and 12.8 hours in female plasma with corresponding AUC determined as 12.4 and 12.2 µg equiv.h/g, respectively.

Recoveries were estimated to be around 85-93% based on cumulative excretion, plus cage wash/cage debris.

Conclusion

^{14}C phenyl labelled isoflucypram (5 mg/kg bw) was rapidly absorbed after oral gavage dosing to rats. Isoflucypram, or its metabolites were not found to be retained to any significant extent in any of the investigated tissues and organs. Elimination of radioactivity was rapid, and the majority of tissues did not contain quantifiable radioactivity at the final sampling time of 168 hours post-dose. Highest radioactivity concentrations, excluding tissues of the gastrointestinal tract, were determined in the liver, renal cortex and renal medulla. No radiolabel was detected in the expired air. Overall, WBAL demonstrated that male and female rats exhibit a very similar absorption, distribution, and excretion with no potential for retention of either unchanged parent and/or metabolites.

([redacted] 2017)

B.6.1.2. Absorption, distribution, metabolism and excretion by other routes

There are no toxicokinetic studies conducted via the dermal or inhalation routes. Although there is no information on uptake across the respiratory tract, as isoflucypram readily crosses the GIT (83.78 and 91.04% of the administered dose) it is reasonable to presume uptake across the respiratory tract will be 100%. Dermal absorption of isoflucypram from its representative product is addressed in CP_B6.

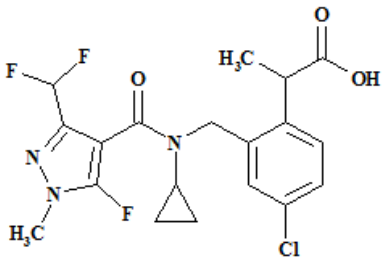
B.6.1.3. Absorption, distribution, metabolism and excretion study of isoflucypram metabolite M12 (isoflucypram carboxylic acid)

Study	[Pyrazolyl-4-^{14}C]BCS-CY26497: Pilot metabolism experiments in male rats
Reference	[redacted] (2017d)
Date performed	28/09/17
Test facility	[redacted]
Report reference	M9992278-6
Guideline(s)	OECD TG 417
Deviations from the guideline	None
GLP	Yes
Test material	pyrazolyl-4-14C]BCS-CY26497 (BCS-CN88460-carboxylic acid)
Study acceptable	Yes

Methods

The absorption, excretion and metabolism of pyrazolyl-4-^{14}C labelled isoflucypram carboxylic acid (M12), one of the major metabolites of isoflucypram, were investigated in male Wistar rats. The rats received a single dose of 5 mg/kg bw of the metabolite by oral gavage, in 0.5% aqueous tragacanth. The elimination of radioactivity from blood was analysed quantitatively by collecting micro samples from one single rat per time-point. In addition, the amount of radioactivity in excreta (urine and/or faeces) was determined for selected time periods in all rats. Single animals were sacrificed at 1, 4, 8, 24, and 48 h after administration for investigation of urine, faeces, plasma, liver and kidney samples were collected. However, the liver and kidney samples were not investigated separately. It is unclear to the RMS why this metabolite was selected for further in-depth investigation.

Figure B.6.1-5: BCS-CY26497 or M12 (position of radiolabel on pyrazolyl-4-^{14}C)



Results

[Pyrazolyl-4-^{14}C] isoflucypram carboxylic acid was absorbed very rapidly from the gastrointestinal tract of male rats after a single oral dose of 5 mg/kg bw. The plasma maximum was reached immediately after dosing (see table below).

Table B.6.1-22: Equivalent and dose normalised concentrations of plasma micro samples from one selected male rat (sacrificed 48 h after dosing) after a single oral administration of 5 mg [pyrazole-4-^{14}C] isoflucypram carboxylic acid /kg

Sampling time [h p admin.]	Equiv. concentration C [µg eq/g]	Dose normalised concentration C_{norm})
0.25	4.7157	0.9246
0.5	4.0252	0.7893
1	2.4024	0.4711
4	0.8195	0.1607
8	0.7936	0.1556
24	0.0355	0.0070
48	0.0469	0.0092

The majority of the radioactivity administered was excreted via the faeces and was 103.68% of dose with around 1.5% of the administered dose found in the urine. Excretion was nearly complete within 24 h post-dose.

In both urine and faeces, unchanged parent compound was the most abundant component (58.6% of dose) and the most abundant metabolite was desmethyl-carboxylic acid or M11 (31.5% of administered dose). Isoflucypram desmethyl-lactic acid or M9 (2.9%) and isoflucypram lactic acid or M10 (2.4%) were also present in smaller amounts.

Table B.6.1-23: Quantitative evaluation of parent compound and its metabolites in urine and faeces after a single oral dose of 5 mg [pyrazole-4-^{14}C] isoflucypram carboxylic acid/kg (Animal 387)

Peak ID	**Metabolite (M code)**	**Route of Elimination**		
		Urine (0 - 24 h)	Faeces (0 - 24 h)	**Total**
		% of dose administered		
U1	pyrazole-amide (M64)	0.03	---	0.03
U3	cyclopropyl-pyrazole-carboxamide-OH-GlucA (M61)	0.04	---	0.04
U4	pyrazole-carboxylic acid (M63)	0.02	---	0.02
U7	cyclopropyl-pyrazole-carboxamide-GlucA (isomer 1 and 2) M60	0.24	---	0.24
U10	pyrazole-carboxylic acid-Ala (M65)	0.49	---	0.49

Peak ID	Metabolite (M code)	Route of Elimination		
		Urine (0 - 24 h)	Faeces (0 - 24 h)	**Total**
		% of dose administered		
U11	N-methyl-pyrazole-carboxylic acid (M50)	0.14	---	0.14
U15	cyclopropyl-pyrazole-carboxamide (M58)	0.04	---	0.04
U18	cyclopropyl-oxy-pyrazole-carboxamide (M59)	0.06	---	0.06
F13	desmethyl-lactic acid (M09)	---	2.86	2.86
F14	desmethyl-hydroxymethyl-carboxylic acid (M16)	---	0.51	0.51
F16	lactic acid (M10)	---	2.39	2.39
U26, F20	desmethyl-carboxylic acid (M11)	0.07	31.40	31.47
U27, F23	BCS-CY26497 (BCS-CN88460-carboxylic acid)	0.04	58.57	58.61
Total identified		**1.18**	**95.72**	**96.89**
U5	unknown	0.04	---	0.04
U12	unknown	0.06	---	0.06
U13	unknown	0.02	---	0.02
U14	unknown	0.01	---	0.01
U17	unknown	0.02	---	0.02
U19	unknown	0.06	---	0.06
U25	unknown	0.02	---	0.02
F32	unknown	---	5.76	5.76
Total characterised		**0.22**	**5.76**	**5.99**
Solids of faeces				1.26
Urine not analysed (24 - 48 h)				0.12
Faeces not analysed (24 - 48 h)				0.94
Total				**105.20**

The principal metabolic reactions of pyrazolyl-4-^{14}C labelled isoflucypram carboxylic acid (M12) in the rat; were; N-demethylation of the pyrazole moiety, hydroxylation of the propionic acid group and defluorination of the difluoromethyl moiety. Cleavage of the phenyl moiety lead to the formation of cyclopropyl-pyrazole-carboxamide compounds, and cleavage of the phenyl moiety in combination with cleavage of the cyclopropyl ring lead to formation of a pyrazole-amide compound and a carboxylic acid compound. The most prominent Phase II reaction was again, glucuronidation.

Conclusion

From the information available, it is unclear whether absorption was complete, as nearly 60% of the administered dose was recovered as the administered substance in the faeces. As only 1.5% of the administered radioactivity was detected in the urine, it is unclear whether there is any significant post hepatic systemic exposure to the administered substance and/or metabolites. The most prominent metabolite identified was desmethyl carboxylic acid with 31.4% of the administered dose detected in the faeces. No unique rat metabolites were identified, compared to administration of isoflucypram. Although there is no specific information on site of metabolism, it is reasonable to conclude the liver is the principal site, given little radiolabel was detected in the urine and the toxicokinetic studies conducted with isoflucypram demonstrated significant metabolite levels in the liver. Elimination was almost exclusively via the faeces.

([redacted] *2017d)*

B.6.1.4. Toxicokinetic information from toxicodynamic studies

Some limited toxicokinetic information is available from standard toxicological studies on isoflucypram. The plasma levels of isoflucypram and two metabolites, desmethyl carboxylic acid or M11 and BCS-CX99798 (N-cyclopropyl-3-(difluoromethyl)-5-fluoro-1H-pyrazole-4-carboxamide) or M58 were determined in rat and mice lifetime studies, in the rat developmental toxicity study and in the 12-month dog repeated dose study. The latter metabolite represents a cleavage product of isoflucypram. More limited measurements of plasma levels of isoflucypram only were made in the 90-day dog study. Furthermore, 4 additional metabolites were measured in plasma after 24 months of dosing in the rat chronic study (M01, M02, M06, M12). The methods of analysis for these plasma measurements were fully validated (see document CA_B5 for further details).

2-year rat, via the diet

Rats (50/sex/group) were treated in the diet with 0, 30, 150, 450 (m) or 800 ppm (f) isoflucypram for 2 years. Mean plasma concentrations of isoflucypram were measured at 3-4 months, 12 and 24 months, whilst the plasma concentrations of metabolites M11 and M58 were measured at 12 and 24 months. In addition, metabolites M01, M02, M06 and M12 were measured after 24 months of treatment. Parent compound was consistently detected at relatively low levels at all time-points; dose-related increases of all metabolites were generally observed (plasma concentrations of the metabolites were greater in females than in males). Levels of these metabolites in plasma were significantly higher than those of the parent.

Table B.6.1-24: Plasma concentrations of isoflucypram and metabolites in the 2-year rat study

	Males			**Females**		
Dose-levels (ppm)	**30**	**150**	**450**	**30**	**150**	**450/800**
3/4 months						
Isoflucypram (mg/l)	< LOQ*	< 0.010	< 0.019	< LOQ	< 0.012	0.015
12 months						
Isoflucypram (mg/l)	< 0.01	< 0.015	< 0.015	< 0.01	0.014	0.068
Desmethyl carboxylic acid (M11) (mg/l)	0.074	0.320	0.523	0.155	0.423	0.813
BCS-CX99798 (M58) (mg/l)	0.020	0.123	0.377	0.015	0.143	1.278
24 months						
Isoflucypram (mg/l)	< 0.010	< 0.01	0.032	< 0.01	0.017	0.027
Desmethyl carboxylic acid (M11) (mg/l)	< 0.015	0.069	0.310	0.012	0.083	1.230
BCS-CX99798 (M58) (mg/l)	0.111	0.540	0.895	0.121	0.682	1.470
M01 (mg/l)	< LOQ	0.015	0.033	< LOQ	0.029	0.026
M02 (mg/l)	< LOQ	0.047	0.21	0.07	0.050	0.20
M06 (mg/l)	< LOQ	0.077	0.140	0.015	0.180	0.44
M12 (mg/l)	< LOQ	0.049	0.078	0.06	0.025	0.11

*limit of quantification - 0.01 mg/L

18-month mouse – via the diet

Mices (50/sex/group) were treated in the diet with 0, 50, 250 or 1250 ppm isoflucypram for 2 years. Mean plasma concentrations of isoflucypram were measured at 3 and 12 months, of metabolite M58 at 18 months whilst the plasma concentrations of metabolite M11 were measured at 12 and 18 months. Parent compound was detected at

levels only slightly above the LOQ in both sexes with plasma concentrations being greater in females than in males. Levels of these two metabolites in plasma were significantly higher than those of the parent.

Table B.6.1-25: Plasma concentrations of isoflucypram and metabolites M58 and M11 in the 18-month mouse study

	BCS-CN88460, dietary concentration in ppm					
	Males			**Females**		
3 months	**50**	**250**	**1250**	**50**	**250**	**1250**
Isoflucypram (mg/l)	< LOQ	< LOQ	< 0.0102	< LOQ	< LOQ	< 0.0184
12 months						
Isoflucypram (mg/l)	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< 0.0198
BCS-CX99798 or M58 (mg/l)	0.0294	0.125	0.486	0.0414	0.312	0.986
Desmethyl carboxylic acid or M11(mg/l)	0.0532	0.156	1.21	0.0692	0.590	6.50
18 months						
Isoflucypram (mg/l)	< LOQ	< LOQ	< 0.0106	< LOQ	< LOQ	< 0.0254
BCS-CX99798 or M58 (mg/l)	0.0334	0.134	0.584	0.0656	0.356	0.844
Desmethyl carboxylic acid or M11 (mg/l)	0.0610	0.198	1.54	0.0638	0.552	4.40

*limit of quantification - 0.01 mg/L

Rat developmental toxicity study – gavage dosing

Dosing was via oral gavage on days 6-20 of gestation at 0, 25, 125, and 625 mg/kg bw/day, in aqueous 0.5% methylcellulose 400. The plasma concentration of isoflucypram and its metabolites M58 and M11 were measured at the end of the study in five animals from each group. The concentration of isoflucypram itself was only marginally above the limit of quantification at all doses, although there was a dose-related increase in the concentrations of the two metabolites, indicating that the test item had been absorbed and extensively metabolized after administration. Levels of these two metabolites in plasma were significantly higher than those of the parent.

Table B.6.1-26: Mean concentrations of isoflucypram and the metabolites M58 and M11 in plasma at necropsy

	Isoflucypram dose in mg/kg bw/day			
	0	**25**	**125**	**625**
Isoflucypram (mg/l)	< LOQ*	< 0.012	0.015 ± .007	0.017 ± 0.003
BCS-CX99798 or M58 (mg/l)	< LOQ	0.039 ± 0.020	0.147 ± 0.037	0.676 ± 0.260
Desmethyl carboxylic acid or M11 (mg/l)	< LOQ	0.339 ± 0.197	0.273 ± 0.106	0.607 ± 0.306

*limit of quantification - 0.01 mg/L

Dogs 90-days

Dogs (4/sex/group) were treated via the diet with 0, 170, 500 or 1500 ppm isoflucypram for 90 days. At the end of the study a single blood sample was collected from all surviving animals in each treated group before feeding, and approximately 4 and 7 hours after feeding and analysed for parent isoflucypram only. Concentrations of isoflucypram in the blood (measured in week 12) were found to increase in a dose-related manner, with the greatest concentration being observed at 4 hours post-feed (in each treated group); at the pre-feeding measurement, low concentrations that were either below or only slightly above the LOQ were observed (see table below). Comparison of the pre-feeding and post feeding data indicate that the previous dose had been metabolised and eliminated from the plasma.

Table B.6.1-27: Blood plasma concentrations of isoflucypram in the 90-day dog study

Dose levels (ppm)	Males				Females			
	0	**170**	**500**	**1500**	**0**	**170**	**500**	**1500**
Pre-feeding	< LOQ*	< LOQ	< 0.012	< 0.027	< LOQ	< LOQ	< LOQ	0.040
4h after feeding	-	0.053	0.184	1.528	-	< 0.048	0.147	1.280
7h after feeding	-	0.036	0.124	1.030	-	< 0.044	0.119	0.825

*limit of quantification - 0.01 mg/l

Dogs – 12 Months

Dogs (4/sex/group) were treated via the diet with 0, 150, 600 or 1800 ppm isoflucypram for 12 months. After 90 days of dosing a single blood sample was collected from all surviving animals before feeding, and approximately 4 and 7 hours after feeding and plasma concentrations of isoflucypram and its metabolites M58 and M11 determined. The greatest concentration of isoflucypram was noted 4-hours after dietary administration, with the lowest (below or slightly above the LOQ) being noted at the pre-treatment measurement. A similar pattern was observed for the metabolites. Comparison of the prefeeding and post feeding data indicate that the previous dose had been metabolised and eliminated from the plasma.

Table B.6.1-28 Plasma concentrations of isoflucypram and metabolites M58 and M11 in the 12-month dog study

Dose levels (ppm)							
	Time-point	**Males**			**Females**		
		150	**600**	**1800**	**150**	**600**	**1800**
Isoflucypram	*Pre-feeding*	< LOQ*	< 0.013	< 0.048	< LOQ	< 0.016	0.145
	4h after feeding	< LOQ	0.108	2.540	< 0.02	0.225	1.365
	7h after feeding	< 0.011	0.115	1.795	< LOQ	< 0.231	0.603
BCS-CX99798 (M58)	*Pre-feeding*	< LOQ	0.031	< 0.093	< LOQ	0.037	0.087
	4h after feeding	< LOQ	0.052	0.193	< 0.01	0.077	0.140
	7h after feeding	< 0.012	0.074	0.225	< 0.010	0.088	0.176
Desmethyl carboxylate (M11)	*Pre-feeding*	< LOQ	0.129	0.114	< 0.01	0.101	0.183
	4h after feeding	< 0.011	0.225	0.553	< 0.020	0.305	0.385
	7h after feeding	< 0.018	0.225	0.433	< 0.012	0.254	0.380

*limit of quantification - 0.01 mg/lL

B.6.1.5. *In vitro* comparative metabolism investigation with Pyrazole-4-^{14}C labelled isoflucypram

Study	[Pyrazole-4-^{14}C]BCS-CN88460 Metabolic Stability and Profiling in Liver Microsomes from Different Animals and Humans for Inter-species Comparison
Reference	Lagojda, A and Doebbe, A. (2017)
Date performed	24/08/17
Test facility	Bayer AG BAG-CS-EnSa-Testing 40789 Monheim Germany
Report reference	M9992283-2
Guideline(s)	No
Deviations from the guideline	NA
GLP	Yes
Test material	pyrazolyl-4-^{14}C]BCS-CY26497 (BCS-CN88460-carboxylic acid)
Study acceptable	Yes

Methods

The *in-vitro* metabolite profile of 6.6 µM and 13.2 µM ^{14}C-labelled isoflucypram was determined after incubation with liver microsomes from humans (pooled male and female) rats (separate male and female) mice (separate male and female) and male dogs and rabbits. Given there can be sex differences in xenobiotic metabolic capacity, it is unclear why some microsomes were pooled and whether this is considered to make a difference to the findings. No metabolites were specifically identified as the sample size was too small. Comparison between samples was on the basis of HPLC profiles.

The incubation system consisted of a phosphate buffer (pH 7.4) and the liver microsomes of humans, rats, mice, dogs and rabbits with a protein concentration of 0.5 mg protein/mL. 6.6 or 13.2 µM [pyrazole-4-^{14}C] isoflucypram was incubated separately with the different liver microsomes in the incubation buffer. The enzymatic activity was started by addition of the NADPH regeneration system and stopped after 20, 40 or 60 minutes by addition of 100 µL acetonitrile. All incubations were performed at 37 ± 1°C. Control incubations were conducted without liver microsomes or without NADPH generating biochemical system in order to show the stability of the test compound in the incubation system. The total number of metabolites was identified by HPLC.

Results

The mass balance of each sample was between 67 and 112% recovered in the supernatants following centrifugation of the incubated suspensions. Liver microsomes were metabolically active as demonstrated by the conversion of ^{14}C testosterone to a range of metabolites. Decreasing amounts of testosterone with increasing formation of radioactive metabolites (up to 15) demonstrated sufficient metabolic capability of the liver microsomes used in this study.

Human microsomes metabolised 6.6 µM isoflucypram to some extent, with around 65% unchanged isoflucypram remaining after 60 minutes but extensively metabolised the 13.2 µM sample with only 16% unchanged parent remaining after 60 minutes (see table B.6.1-29 below). It is possible these findings represent the existence of two enzymes, one low capacity/high affinity and the other high capacity/low affinity.

In comparison to human microsomes (34% conversion after 60 minutes), male rat microsomes (96.8% conversion at 60 minutes) were more efficient in clearing the low concentration of isoflucypram but conversion of the high concentration was not that different (83.7% in human microsomes compared to 98% in male rats). Female rat microsomes were more efficient in converting isoflucypram, compared to human microsomes (34.2% conversion in humans compared to73.1% in female rats). However, at the higher concentration, human microsomes were more efficient in converting isoflucypram than female rats (83.7% in human microsomes to 62.7% in female rats).

Comparing human microsomes with other species (mice, dogs and rabbits), human microsomes converted 34% of the available isoflucypram after 60 minutes (6.6 µM) but the other species converted 88-96%. However at the higher concentration (13.2 µM) human microsomes converted around 84%, slightly less than mice, dogs and rabbits (92-98%). It was also noted that some sex differences were apparent in mouse microsomes after 20-40 minutes but not after 60 minutes.

Table B.6.1-29: Metabolic conversion of 6.6 and 13.2 µM ^{14}C-isoflucypram in liver microsomes

Species/Test system	**Relative amount of ^{14}C-isoflucypram in the radiochromatogram [%]**			**conversion of isoflucypram (%)**		
Incubation period (minutes)	20	40	60	20	40	60
Human – 6.6 µM						
mixed gender	99.26	47.95	65.23	0	51.7	34.2
Human – 13.2 µM						
mixed gender	39.11	31.40	16.19	60.6	68.3	83.7
control incubation	---	---	99.18	---	---	---
Rat – 6.6 µM						
male	98.93	82.93	3.17	0.3	16.4	96.8
female	52.01	40.97	26.69	47.5	58.7	73.1
Rat – 13.2 µM						
male	7.24	2.98	1.96	92.7	97.0	98.0
control incubation	---	---	99.24	---	---	---
female	57.31	45.01	36.94	42.2	54.6	62.7
control incubation	---	---	99.16	---	---	---
Mouse – 6.6 µM						
male	50.34	5.83	7.94	49.2	94.1	92.0
female	66.94	54.54	11.49	32.5	45.0	88.4
Mouse – 13.2 µM						
male	7.14	1.96	1.82	92.8	98.0	98.2
control incubation	---	---	99.16	---	---	---
female	7.64	8.89	1.65	92.3	91.0	98.3
control incubation	---	---	99.16	---	---	---
Dog – 6.6 µM						
male	87.28	17.92	4.40	11.9	81.9	95.6
Dog – 13.2 µM						
male	39.23	21.57	7.37	60.4	78.2	92.6
control incubation	---	---	99.10	---	---	---
Rabbit – 6.6 µM						
male	57.01	54.74	8.34	42.4	44.7	91.6

Rabbit – 13.2 µM						
male	1.85	2.25	4.95	98.1	97.7	95.0
control incubation	---	---	99.05	---	---	---

The metabolic transformation rate of the test compound [pyrazole-4-^{14}C] isoflucypram over time was calculated according to following equation:

$$\%\ \text{Metabolic transformation rate} = 100 - \left(\frac{(\%\ \text{Area test compound 20, 40 or 60 minutes})}{\%\ \text{Area test compound of control incubations after 60 minutes}} \text{ x } 100\,\% \right)$$

Where the relative percentages of the peak area of the test compound after 20, 40 and 60 minutes incubation time was compared to the relative percentage measured in the control incubations which were exemplarily performed with liver microsomes incubated at 13.2 µM ^{14}C-isoflucypram. The RMS does not consider these values to be a rate, as there is no unit for time.

Inspection of metabolic profiles found that higher numbers of metabolites were detected in liver microsomes from rat (21) mouse (21) and dog (16), compared to rabbit (12) and human microsomes (14). No unique human metabolites were detected. However, as metabolites were not identified in this study, it is possible that HPLC peaks with similar retention times could represent different molecules.

Conclusion

This *in vitro* comparative metabolism study indicates that although pooled human microsomes metabolised isoflucypram less rapidly than other species (rats, mice, rabbits and dog) they did not produce any metabolites that were not observed in rats, mice and dogs, the main species for which metabolism data are available.

Overall, the metabolic profile in all liver microsome incubations was qualitatively similar, but human microsomes were less efficient at metabolising isoflucypram, particularly in comparison to the mouse, rat and rabbit.

(Lagojda, A and Doebbe, A, 2017)

B.6.1.6. Summary of toxicokinetics

The toxicokinetics of isoflucypram have been investigated in rats via the oral route only, following gavage dosing. The available studies comprise two ADME investigations including bile duct-cannulation and two whole body autoradiography experiments using isoflucypram, separately radiolabelled at the pyrazole and phenyl moieties and a comparative *in vitro* metabolism study (including human liver microsomes). None of these studies were conducted to OECD TG, but were GLP compliant and are considered sufficiently robust to provide information on the toxicokinetics in the rat following oral dosing by gavage Limited additional information is also available on blood levels of isoflucypram, and a number of metabolites from repeated dose studies in rats, mice and dogs and a developmental toxicity study in rats dosed via the oral route only. No toxicokinetic studies are available by other routes of exposure. Limited additional toxicokinetic information on a metabolite, isoflucypram carboxylate (M12) is also available.

Isoflucypram

Absorption

From the available information, isoflucypram is rapidly and well absorbed from the gastrointestinal tract (84 and 88% in males and females respectively). Although uptake from the GIT is extensive, it appears that post-hepatic systemic exposure to unchanged isoflucypram and/or its metabolites accounts for around 15% of the administered dose, as no more that 15% was detected in the urine and a significant amount is excreted in bile within 24 hours. Time to maximal plasma concentration (Tmax) was 1 hour with a maximum concentration of around 0.5µg/ml. There is no evidence of any significant sex differences in absorption. However, absorption of isoflucypram from the GI tract does appear to be saturable. Overall**, an oral absorption** value of **100%** is proposed. For **post-hepatic systemic availability,** the RMS proposes a value of **15%**.

Dermal absorption of isoflucypram from its representative product is 2% and 5% for the concentrate and dilution respectively (further details are available in document CP_B6). There are no data to determine the inhalation

absorption of the substance. However, based on the extensive oral absorption, a default inhalation absorption value of 100% can be assumed.

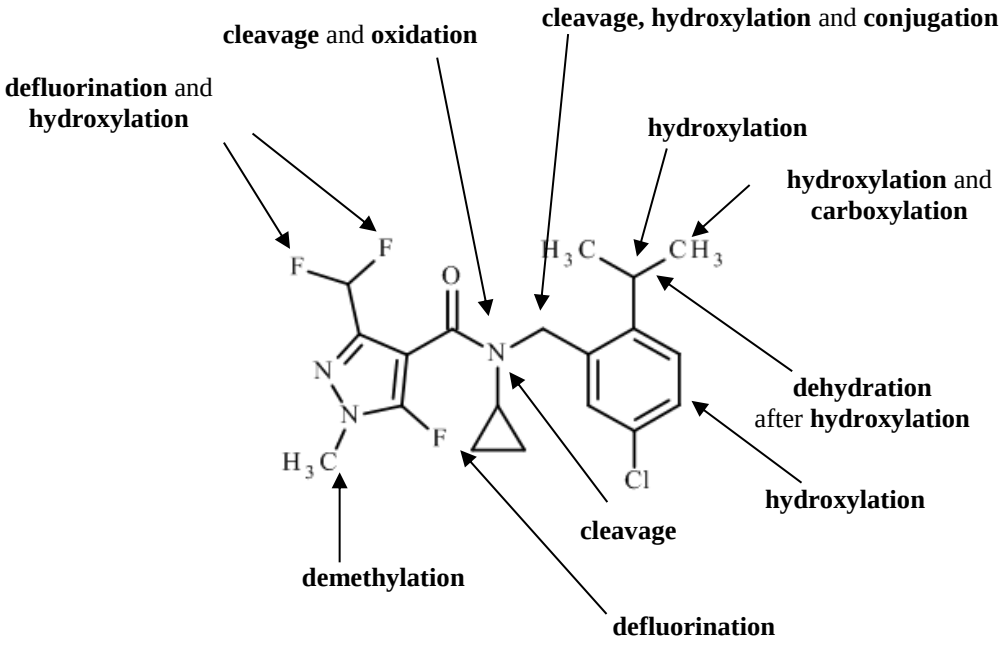
Distribution

Distribution of radiolabelled isoflucypram and/or its metabolites was predominately to the organs of metabolism and excretion, the liver and kidneys. There was no evidence of retention in any organs/tissues. Although blood levels of individual metabolites were not measured, very low levels of total radioactivity were found in terminal blood samples (below 0.1% of the administered dose) 72 hours after dosing.

Limited information on plasma levels of isoflucypram and a number of metabolites, M01, M02, M06, M11, M12 and M58 from repeat dose studies in rats, mice and dogs and from the developmental toxicity study in rats confirmed that there is little or no detectable unchanged isoflucypram in blood plasma following repeated exposure. The levels of the metabolites in plasma were significantly higher than those of the parent.

Metabolism

In vivo toxicokinetic investigations of metabolism found that isoflucypram was rapidly and extensively metabolised such that there was no significant, post-hepatic exposure to unchanged isoflucypram. Studies with ^{14}C pyrazole labelled isoflucypram demonstrated that, in the rat, this moiety was metabolized in the liver principally via N-demethylation of the pyrazole methyl and/or oxidation of the isopropyl group to desmethyl carboxylates or lactate. Investigation of plasma metabolites found desmethyl carboxylic acid (M11) to be the most abundant metabolite. The Phase 1 metabolites act as substrates for a number of Phase II reactions, principally, glucuronidation. There were no significant sex differences in metabolism. Studies with ^{14}C-phenyl labelled isoflucypram found metabolism to be largely similar to that of the ^{14}C pyrazole labelled isoflucypram. However, some benzyl alcohol-related compounds were identified, indicating cleavage of the parent isoflucypram, although this only represents a minor metabolic pathway. No individual metabolite nor the parent compound was found in urine or plasma at levels greater than 10% of the administered dose. Analysis of plasma levels of isoflucypram and a number of metabolites occurring in plants (M01, M02, M06, M11, M12 and M58) from the rat chronic study showed that the levels of these metabolites were significantly higher than those of the parent compound. The principal metabolic reactions and sites for isoflucypram are detailed in the diagram below:



A comparative *in vitro* metabolism study indicated that although pooled human microsomes metabolised isoflucypram less rapidly than other species (rats, mice, rabbits and dog) they did not produce any metabolites that were not observed in the rat or dog, the main species for which most toxicological information is available.

Excretion

Excretion of most metabolites was via the bile, accounting for around 77.6-85.5 % of the administered dose, in males and females respectively. Investigations in bile-duct cannulated rats found no unchanged parent in the bile;

therefore, any unchanged parent in the faeces is considered to represent unabsorbed material (20.7% and 15.5% in males and females respectively). Urinary excretion accounted for 8 – 13 % of the total administered dose, with no individual metabolite present above 10%. No radiolabel was detected in the expired air. The radioactivity retained in the carcass accounted for no more than 0.5% of the administered dose.

Although there is no toxicokinetic information from other relevant routes of exposure, given the significant first-pass effect, quantitative differences in the degree of systemic exposure to unchanged isoflucypram and metabolites would be anticipated following inhalation or dermal exposure.

Isoflucypram carboxylic acid (M12)

A limited toxicokinetic study was also conducted in rats by gavage with pyrazolyl-4-^{14}C labelled isoflucypram carboxylic acid (M12), a prominent isoflucypram metabolite. From the information available, it is unclear whether absorption of the isoflucypram carboxylic acid was complete, as nearly 60% of the administered dose was recovered as unchanged parent in the faeces. This is not unexpected as the presence of the carboxylate group will increase the polarity of the molecule, making it less likely to cross plasma membranes unless facilitated by an active transport process.

The most prominent metabolite of M12 was desmethyl carboxylic acid (M11) with 31.4% of the administered dose found in the faeces and 0.07% found in the urine. As around 1.5% of the total administered radioactivity was detected in the urine, it is uncertain whether there is any significant post-hepatic systemic exposure to unchanged M12. Elimination of most of the absorbed dose was via the faeces.

B.6.2. ACUTE TOXICITY

The acute toxicity of isoflucypram technical has been investigated *via* the oral, dermal and inhalation routes. An *in vivo* study has been performed to investigate skin irritation while eye irritation has been investigated sequentially *in vitro* and then *in vivo*. Skin sensitisation has been investigated in a murine local lymph node assay. All of these studies were conducted according to standard OECD Test Guidelines and are GLP compliant.

B.6.2.1. Oral

Study	BCS-CN88460 technical - Acute oral toxicity study in the rat (up and down procedure)
Reference	[redacted] (2014a)
Date performed	26 February-25 March 2014
Test facility	[redacted]
Report reference	14/069-001P
Guideline(s)	OECD No.: 425, adopted October 2008
Deviations from the guideline	None
GLP	Yes. Signed QA and GLP certificates provided
Test material	isoflucypram technical. Batch: 2013-006492, Purity 94.2% w/w, CAS No 1255734-28-1.
Study acceptable	Yes

The potential for isoflucypram to cause acute oral toxicity was investigated in rats in a OECD TG compliant study following the Up and Down Procedure.

After overnight fasting, 5 female Wistar rats were sequentially administered a single dose of isoflucypram in PEG40 by oral gavage at a dose level of 2000 mg/kg bw. All animals were observed after dosing at 30 minutes and 1, 2, 3, 4, and 6 hours after treatment, and then once each day for 14 days. Body weight was measured the day before and the day of dosing and weekly thereafter. At the end of the observation period, animals were necropsied.

No mortalities, clinical signs, effects on body weight or body weight gain, or macroscopic findings were observed in any animal following oral gavage administration of isoflucypram to female rats.

Under the conditions of this study, the acute oral LD_{50} of isoflucypram was greater than 2,000 mg/kg of body weight in female rats. Isoflucypram is not acutely toxic by the oral route and, in accordance with Regulation (EC) 1272/2008, does not meet the criteria for classification for acute oral toxicity or STOT-SE.

(2014a)

B.6.2.2. Dermal

Study	BCS-CN88460 technical - Acute dermal toxicity study in rats
Reference	[redacted] (2014b)
Date performed	19 February-12 March 2014
Test facility	[redacted]
Report reference	14/069-002P
Guideline(s)	OECD 402 (1987)
Deviations from the guideline	None
GLP	Yes. Signed QA and GLP certificates provided
Test material Vehicle	isoflucypram technical. Batch: 2013-006492, Purity 94.2% w/w, CAS No 1255734-28-1. PEG40
Study acceptable	Yes

The potential of isoflucypram to cause acute dermal toxicity was investigated in rats in a guideline compliant study.

Isoflucypram technical was applied on moistened gauze to the backs of 10 Wistar rats (five male and five female) at a dose of 2000 mg/kg bw for 24 hours under semi-occlusive conditions. Clinical observations were performed on all animals at 1 and 5 hours after dosing and daily for 14 days thereafter. Body weight was measured prior to compound application on study day 0 and on study days 7 and 14. Rats were euthanized and subjected to a gross macroscopic examination on study day 14.

There were no mortalities, systemic clinical signs, or treatment-related effects on body weight or body weight gain, nor were there any abnormalities noted at necropsy. Very slight erythema (score 1) was noted in five of 10 animals (two males and three females) on study day 1 only. All local signs observed were reversible by study day 2.

Under the conditions of this study, the acute dermal LD_{50} of isoflucypram was greater than 2,000 mg/kg body weight in rats. Isoflucypram is not acutely toxic by the dermal route and, in accordance with Regulation (EC) 1272/2008, does not meet the criteria for classification for acute dermal toxicity or STOT-SE.

([redacted] 2014b)

B.6.2.3. Inhalation

Study	BCS-CN88460 technical - Acute inhalation toxicity study (nose-only) in the rat
Reference	[redacted] (2014)
Date performed	10 April-27 June 2014
Test facility	[redacted]
Report reference	14/069-004P
Guideline(s)	OECD 403 (2009)
Deviations from the guideline	None that would impact on the validity of the study.
GLP	Yes. Signed QA and GLP certificates provided
Test material	10-20% (w/w) isoflucypram technical with acetone. Batch: 2013-006492, Purity 94.2% w/w, CAS No 1255734-28-1.
Study acceptable	Yes

An acute inhalation toxicity study was conducted with rats to determine the potential for isoflucypram to produce toxicity from a single concentration *via* the inhalation route.

Male and female Wistar rats (5/sex/group) were exposed, nose-only, to a liquid aerosol atmosphere of isoflucypram (diluted to 10-20% w/w with acetone then aerosolized) at concentrations of 1.03, 2.04 and 2.87 mg/l for four hours.

Clinical observations were performed for all animals during exposure at hourly intervals, following removal from restraint, approximately 1 hour following the end of exposure, and daily for 14 days thereafter. Body weight was measured on study day 0 before the exposure, and on study days 1, 3, 7, and 14 or at death. Gross necropsy was performed on all animals sacrificed on study day 14.

The test atmospheres were sampled from the breathing zone during each exposure period, 4-18 times at approximately equal intervals. Analysis of the particle size distribution of the aerosol at the animals' breathing zone demonstrated that the test atmosphere was respirable. The mean achieved actual concentrations and nominal concentrations, and the mean values of the particle size distribution parameters calculated from two or three samples of each exposure are presented in Table B.6.2.1. The method of analysis used to determine the achieved test concentrations was based on a rather crude approach (weight of the material on the filter) and is considered to be not fully validated (see document CA_B5 for further details). However, as hazard classification for acute inhalation toxicity (see below) has already been proposed on the basis of this investigation, repetition of such vertebrate study with a fully validated method of analysis is not justified.

Table B.6.2-1: Mean achieved actual and nominal aerosol concentration, MMAD, GSD, and respirable fraction (% < 4 µm) for each treatment group

Parameter	isoflucypram treatment group			4 (acetone control)
	1	2	3	
Target concentration (mg/l)	1	3	2	0
Mean achieved concentration (mg/l)	1.03	2.87	2.04	0.0
SD of achieved concentration (mg/l)	0.07	0.14	0.14	0.0
MMAD (µm)	1.52	1.41	1.56	
GSD	1.99	1.93	1.88	
Inhalable fraction (% < 4 µm)	92.0	94.3	93.2	

One male and one female died at 2.04 mg/l, while two males and all five females died at 2.87 mg/l during the exposure. Clinical signs observed in animals from all treatment groups surviving the exposure period included noisy or gasping respiration, decreased activity, ataxia (slight to severe), hunched back, slight sneezing, prostration, or coma. All surviving animals were free of clinical signs of toxicity by study day 3 at the latest.

Slight body weight loss or body weight retardation was noted in all treatment groups on day 1. All surviving animals had returned to their initial body weights no later than study day 7.

Dark or red diffuse or multifocal discoloration of the lungs were observed in all animals which were found dead. Among surviving animals, there were no findings of note at necropsy.

Under the conditions of this study, a 4-hr LC_{50} of 2.518 mg/l (aerosol) was calculated for males and females combined (95% confidence limits: 2.010-3.663 mg/l) with 3.131 mg/l for males and 2.209 mg/l for females. Therefore, isoflucypram is acutely harmful by the inhalation route and meets the criteria for classification in category 4 for acute inhalation toxicity (**Acute Tox 4; H332**) in accordance with Regulation (EC) 1272/2008.

([redacted] *2014)*

B.6.2.4. Skin irritation

The RMS requested a justification for performing the *in vivo* skin irritation study. The applicant replied saying that an *in vitro* skin corrosion or irritation study was not conducted prior to the conduct of this *in vivo* skin irritation study. As no indications of corrosion or pain reactions were observed in the dermal toxicity study conducted in the rat, and as the pH of the test substance dissolved in water was measured by the conducting laboratory to be 4.5, it was considered that no conditions existed which would prevent the conduct of the in vivo study. This justification is not accepted as the *in vitro* study should be used instead of the *in vivo* study and not as a precursor. The conduct of this study is therefore considered to be in contravention of Article 62 of Regulation (EC) 1272/2008.

Study	BCS-CN88460 technical - Acute skin irritation study in rabbits
Reference	[redacted] (2014c)
Date performed	21 February-1 March 2014
Test facility	[redacted]
Report reference	14/069-006P
Guideline(s)	OECD 404 (2002)
Deviations from the guideline	None
GLP	Yes. Signed QA and GLP certificates provided
Test material	Isoflucypram technical. Batch: 2013-006492, Purity 94.2% w/w
Study acceptable	Yes. However the study is considered to be in contravention of Article 62 of Regulation (EC) 1272/2008.

In a study to determine its skin irritation potential, 0.5g of isoflucypram was applied to the intact shaved flank of 3 male young adult New Zealand White rabbits. The duration of treatment was four hours under semi-occlusive conditions. The treated skin surface was examined at 1, 24, 48, and 72 hours after patch removal.

There were no observed signs of skin irritation in any of the treated animals at any time point after removal of the test item (all scores for erythema/eschar and oedema were zero). No clinical signs of systemic toxicity were observed in the animals during the study, and no mortalities occurred. The body weights of all rabbits were considered to be within the normal range of variability.

Table B.6.2-2: Individual values for skin irritation in three rabbits treated with isoflucypram for four hours

Animal	Observation	24h	48h	72h	Mean scores	Response	Reversible (days)
00806	Erythema and eschar	0	0	0	0.00	-	N/A
	Edema	0	0	0	0.00	-	N/A
00811	Erythema and eschar	0	0	0	0.00	-	N/A
	Edema	0	0	0	0.00	-	N/A
00810	Erythema and eschar	0	0	0	0.00	-	N/A
	Edema	0	0	0	0.00	-	N/A

Under the conditions of this study, isoflucypram was not irritating to the skin when tested in male New Zealand White rabbits. Isoflucypram does not meet the criteria for classification a skin irritant in accordance with Regulation (EC) 1272/2008.

([redacted] *2014c)*

B.6.2.5. Eye irritation

Ex vivo study

Study	BCS-CN88460 technical – *Ex vivo* eye irritation test in isolated chicken eyes
Reference	Váliczkó, É.; 2014
Date performed	31 March 2014
Test facility	Envigo CRS GmbH, Rossdorf, Germany
Report reference	14/069-005N
Guideline(s)	OECD 438
Deviations from the guideline	None
GLP	Yes
Test material	Isoflucypram technical. Batch: 2013-006492, Purity 94.2% w/w
Study acceptable	Yes

An *ex vivo* eye irritation screening study was conducted in isolated chicken eyes using isoflucypram. A total of three eyes were used, and an amount of 30 mg of the test substance was applied to the center of the cornea of each eye to cover the entire surface of the cornea. After ten seconds, the cornea and entire surface of the eye was then rinsed with saline. Positive control eyes were treated with 30 mg imidazole, while the negative control eye was treated with 30 µL saline (NaCl, 0.9% w/v). All eyes were evaluated before the treatment and at approximately 30, 75, 120, 180, and 240 minutes after the post-treatment rinse. Corneal thickness and corneal opacity were measured at all time points while fluorescein retention was measured on two occasions, at baseline (t=0) and at approximately 30 minutes after the post-treatment rinse. The observations are summarised in the table below.

Table B.6.2-3: Observations in isolated chicken eyes treated with either isoflucypram, imidazole (positive control), or isotonic saline (negative control)

Observation	Isoflucypram Value / ICE Class	Imidazole Value / ICE Class	Phys. saline Value / ICE Class
Mean maximum corneal swelling ≤ 75'	0.0% / I	3.3% / I	0.0% / I
Mean maximum corneal swelling ≤ 240'	0.0% / I	7.1% / II	0.0% / I
Mean maximum corneal opacity	0.00 / I	3.67 / IV	0.00 / I
Mean fluorescein retention	0.50 / I	2.83 / IV	0.00 / I
Other observations	The substance was stuck on all corneal surfaces after the post-treatment rinse. The corneal surface was not cleared by 240 minutes after the post-treatment rinse.		none
Overall ICE Class	3 x I	1x II, 2 x IV	3 x I

After rinsing of the eyes, some of the test material remained stuck to the corneal surface (along with some fluorescein retention in treated eyes). It is not possible to predict the effect that the retention of test material to the corneal surface would have in the *in vivo* situation (blinking might clear the surface of the eye but there is also the potential that the movement of the eye lids would cause abrasion of the corneal surface).

Therefore, although the results from this *ex vivo* study indicate that isoflucpyram technical is unlikely to be *severely* irritating to the eye, the eye irritating potential of isoflucypram cannot be fully predicted from this study. It was concluded therefore that an *in vivo* study was required.

(Váliczkó, 2014)

In vivo study

Study	BCS-CN88460 technical - Acute eye irritation study in rabbits
Reference	[redacted] (2014d)
Date performed	20-29 May 2014
Test facility	[redacted]
Report reference	14/069-005N
Guideline(s)	OECD 405 (2012)
Deviations from the guideline	None
GLP	Yes. Signed QA and GLP certificates provided
Test material	Isoflucypram technical. Batch: 2013-006492, Purity 94.2% w/w
Study acceptable	Yes

An acute eye irritation study was conducted to the relevant OECD TG in male New Zealand White rabbits with isoflucpyram, and the irritant effects were evaluated by the Draize method. An amount of 0.1g of the test item was placed into the conjunctival sac of the left eye of each animal, with the untreated right eye serving as control. The eyes were then examined at 1, 24, 48, and 72 hours after application, with fluorescein staining performed 24 hours before application and 24, 48, and 72 hours after application of the test item.

Conjunctival redness, chemosis, and discharge were noted in all three animals at the 24-hour examination. At 48 hours, all three animals showed a decreased amount of conjunctival redness with no chemosis or discharge noted. All observations had reversed by the 72-hour examination.

Table B.6.2-4: Observations in treated eyes of three rabbits administered isoflucypram in the conjunctival sac of one eye

Animal	Effects	24h	48h	72h	Mean score	Reversible (days)
00978	Corneal opacity	0	0	0	0.00	N/A
	Iritis	0	0	0	0.00	N/A
	Redness conjunctivae	2	1	0	1.00	3
	Chemosis conjunctivae	1	0	0	0.33	2
00987	Corneal opacity	0	0	0	0.00	N/A
	Iritis	0	0	0	0.00	N/A
	Redness conjunctivae	2	1	0	1.00	3
	Chemosis conjunctivae	1	0	0	0.33	2
0979	Corneal opacity	0	0	0	0.00	N/A
	Iritis	0	0	0	0.00	N/A
	Redness conjunctivae	2	1	0	1.00	3
	Chemosis conjunctivae	1	0	0	0.33	2

Under the conditions of this study, isoflucypram was mildly irritant to the rabbit eye. These effects were fully reversible within 72 hours. Based on the scores in this study, isoflucypram does not meet the criteria for classification for eye irritation in accordance with Regulation (EC) 1272/2008.

([redacted] *2014d)*

B.6.2.6. Skin sensitization

Study	BCS-CN88460 technical - Local lymph node assay in the mouse
Reference	[redacted] (2015)
Date performed	12 March-12 May 2014
Test facility	[redacted]
Report reference	14/069-037E
Guideline(s)	OECD 429 (2010)
Deviations from the guideline	None
GLP	Yes. Signed QA and GLP certificates provided
Test material	Isoflucypram technical. Batch: 2013-006492, Purity 94.2% w/w
Study acceptable	Yes

The aim of this study was to determine the skin sensitisation potential of isoflucypram following dermal exposure, to the test item formulated in acetone:olive oil 4:1 (v/v). Five female CBA/J Rj mice per dose and control group; negative controls received the vehicle, positive control received 25% α-hexylcinnamaldehyde (HCA), and four groups which received isoflucypram at 5%, 10%, 25%, or 50%.

The formulations of the test item were applied to the experimental animals (25 µl/ear) on study days 1, 2, and 3. On study day 6, the cell proliferation in the local lymph nodes was measured by incorporation of tritiated methyl thymidine (3H-TdR), and the stimulation index was calculated for each group.

No mortality or systemic toxicity was observed during the study. There were no visual signs of local irritancy at the site of application. A minimal amount of test item precipitate was observed on the ears of the animals in the 50% dose group on study days 1-6 and in the 25% dose group on study days 1-3. Alopecia was recorded in the 50% dose group on study days 3-6. Slightly enlarged lymph nodes were recorded (subjective judgement based on observations in previous experiments) for animals in the 50% dose group, and slightly larger than normal lymph nodes were noted in the positive control groups.

No treatment-related effects were observed on body weights in any group. The observed stimulation index was 1.2, 1.2, 2.5, and 5.6 at concentrations of 5%, 10%, 25%, and 50%, respectively. The calculated EC3 value was 29.0%.

Table B.6.2-5: Mean ear thickness, biopsy weight, DPN, and Stimulation Index in the mouse local lymph node assay

Parameter	Day	Ear	Treatment group					
			AOO	5%	10%	25%	50%	HCA
Ear thickness, mm	1	Right	0.22	0.21	0.19	0.21	0.21	
		Left	0.21	0.21	0.21	0.22	0.21	0.20
	3	Right	0.22	0.23	0.23	0.23	0.29	0.23
		Left	0.22	0.23	0.22	0.23	0.28	0.24
	6	Right	0.22	0.22	0.21	0.22	0.24	0.24
		Left	0.22	0.22	0.22	0.22	0.24	0.24
Biopsy weight[1], mg			15.44	15.72	14.45	15.62	16.36	16.01
Group DPN			178.3	207.8	206.5	449.8	997.7	2450.8
Stimulation Index			1.0	1.2	1.2	2.5**	5.6**	14.3**

[1] General historical control range: 11.92-22.53mg. Positive response is > 28.16mg.
** statistically significant at $p < 0.01$.

Under the conditions of the study, isoflucypram was shown to have sensitization potential in the mouse Local Lymph Node Assay, with an EC_3 value of 29.0%. These findings support classification as a category 1B skin sensitiser (**Skin Sens 1B ; H317**) under Regulation (EC) No 1272/2008.

([redacted] *2015)*

B.6.2.7. Phototoxicity

Isoflucypram does not absorb electromagnetic radiation in the range 290-700 nm but has an ultraviolet/visible molar extinction/absorption coefficient that exceeds 10 L x mol^{-1} x cm^{-1}. Therefore, although a phototoxicity study is not strictly required, one was performed anyway.

Study	BCS-CN88460 technical: Cytotoxicity assay *in vitro* with BALB/c 3T3 Cells: Neutral red (NR) test during simultaneous irradiation with artificial sunlight
Reference	Spohr, C.; 2018
Date performed	5 October 2017
Test facility	Envigo CRS GmbH, Rossdorf, Germany
Report reference	GT41JH
Guideline(s)	OECD 432
Deviations from the guideline	None
GLP	Yes. Signed QA and GLP certificates provided
Test material	Isoflucypram technical. Batch: 2013-006492, Purity 94.2% w/w
Study acceptable	Yes

The phototoxicity of isoflucypram was investigated *in vitro* using BALB/c 3T3 cells. The experiment was performed twice. The first experiment served as a range finding experiment (RFE); the second as the main experiment (ME). In both experiments cells were exposed to isoflucypram for one hour at concentrations in the range of 0.49- 62.50 µg/ml. Following exposure to isoflucypram, one group of cells treated with the test item was irradiated with artificial sunlight for 50 minutes with 1.65 mW/cm2 UVA (RFE) and 1.8 mW/cm2 UVA (ME), resulting in an irradiation dose of ~ 5 J/cm2 UVA. A second test group were kept in the dark for 50 minutes. Cytotoxicity was determined by the measurement of Neutral Red. The test included appropriate positive (chlorpromazine) and solvent controls.

The results of the study are summarized in table B.6.2.6 below. In the range finding experiment a dose dependent cytotoxicity was observed after treatment of cells with isoflucypram in the presence and absence of irradiation with artificial sunlight. Based on the results of the range finding experiment isoflucypram showed a possible phototoxic effect on BALB/c 3T3 cells, however this was not confirmed in the main experiment.

Table B.6.2-6: Summary of results of the neutral red assay in the phototoxicity test with isoflucypram (range finding and main experiments)

	Treatment Group	IC50 (+UV) [µg/ml]	IC50 (-UV) [µg/ml]	PIF	MPE	% viability of solvent control of irradiated versus non irradiated plate
RFE	isoflucypram	27.11	28.78	1.062	0.108	94.8
	Positive control	0.596	18.18	30.480	0.568	84.3
ME	isoflucypram	23.44	22.58	0.965	-0.042	89.5
	Positive control	0.4592	11.74	25.576	0.647	99.1

The mean of solvent control values of the irradiated group versus the non-irradiated group met the acceptance criteria. The positive control chlorpromazine induced phototoxicity in the expected range after irradiation with artificial sunlight.

Under the conditions of this study, isoflucypram does not possess any phototoxic potential.

(Spohr, 2018)

B.6.2.8. Summary of acute toxicity

The acute toxicity of isoflucypram technical has been investigated *via* the oral, dermal and inhalation routes. An *in vivo* study has been performed to investigate skin irritation while eye irritation has been investigated sequentially *in vitro* and then *in vivo*. Skin sensitisation has been investigated in a murine local lymph node assay. In addition, phototoxicity was investigated *in vitro*. All of these studies were conducted according to standard OECD Test Guidelines and are GLP compliant. Isoflucypram was of low oral and dermal acute toxicity, but was harmful by the inhalation route (4hr LC_{50} = 2.5 mg/L), thus meeting the criteria for classification with **Acute Tox 4 (H332)** under Reg. (EC) No 1272/2008. It was not a skin or eye irritant, but gave a positive response in the LLNA, triggering classification with **Skin Sens 1B (H317)** under Reg. (EC) No 1272/2008. Isoflucypram was not phototoxic *in vitro*, even though a study was not strictly required. The table below provides an overview of the available acute toxicity studies.

Table B.6.2-7 Summary of isoflucypram acute toxicity data, with classification according to Regulation (EC) No 1272/2008

Study	Result	Reference	Classification according to Reg. (EC) No 1272/2008
Acute oral rat	Oral LD_{50} > 2000 mg/kg bw	[redacted] 2014a	No classification
Acute dermal rat	Dermal LD_{50} > 2000 mg/kg bw	[redacted] 2014b	No classification
Acute inhalation rat	Inhalation 4hr LC_{50} = 2.518 mg/L (both sexes combined) 3.131 mg/L (males) 2.209 mg/L (females)	[redacted]2014	**Acute Tox 4, H332** Harmful if inhaled
Skin irritation, rabbit	Negative	[redacted] 2014c	No classification
Eye irritation *in vitro*, isolated chicken eyes	Neither severe irritant nor non-irritant	Valiczko, 2014	Inconclusive
Eye irritation, rabbit	Negative	[redacted] 2014d	No classification
Skin sensitization (LLNA)	Sensitizing EC3 = 29.0%	[redacted] 2015	**Skin Sens 1B, H317** May cause an allergic skin reaction
Phototoxicity	Negative	Spohr, 2018	Not applicable

In conclusion, isoflucypram is classified for acute toxicity 4 via inhalation (but not by the oral or dermal route) and skin sensitisation 1B. It is most likely that the higher acute inhalation toxicity of isoflucypram compared to the oral route is due to the lack of a first-pass effect by the liver. The low acute dermal toxicity is most likely the consequence of the limited dermal absorption of the substance.

B.6.3. SHORT-TERM TOXICITY

The short-term toxicity of isoflucypram has been investigated in rats, mice and dogs via the oral (dietary) route of exposure (28- and 90-day studies); a one-year study in dogs is also available. The available 28-day studies were intended as range-finding studies for the succeeding 90-day investigations, and as such were not performed according to GLP or to any particular guidelines; nevertheless, the RMS considers that these studies were well-conducted and sufficiently reliable to contribute to the overall picture of the repeated-dose toxicity of isoflucypram. No other routes of exposure have been investigated.

The main target organ of toxicity in all species is the liver. The toxicological significance of the effects on the liver has been assessed by the RMS using a weight-of-evidence approach, with a clear distinction being made between effects that are clearly adverse and those which are potentially adaptive. This has been carried out in line with the TAB (Technical Agreements for Biocides) entry[1], agreed at the Biocide WG-IV-2018 meeting (WGIV2018_TOX_6-2); a paper which is based on several international reviews of liver effects (JMPR 2006 and 2015) and which describes a weight-of-evidence approach for the evaluation of liver effects in repeated-dose toxicity studies. Hepatocellular hypertrophy is typically related to increased functional capacity of the liver which allows the maintenance of homeostasis in the organism after xenobiotic exposure. A general increase in the size of the liver is observed (owing to cell enlargement and fluid accumulation); this is considered a potentially beneficial, adaptive response. However, there is the potential that the capacity of the homeostatic mechanisms may be exceeded and in these cases the organism would be unable to return to its previous state once exposure has ended (thus constituting an adverse response). Hypertrophy as an adaptive response should not be accompanied by adverse histopathology (necrosis, apoptosis, pigment deposition or hyperplasia), or by substantial changes in clinical chemistry indicative of liver toxicity (decreased albumin or increased activities of ALT, AST, ALP, GGT, bilirubin or cholesterol). In line with the TAB entry, relative liver weight increases up to 15%, which are not accompanied by other signs of liver dysfunction will be considered by the RMS to be an adaptive rather than an adverse response.

B.6.3.1. Oral 28-day studies

B.6.3.1.1 Oral 28-day study in rats

A 28-day dietary toxicity study has been conducted in rats. This range-finding study was not performed to any particular guideline, but was broadly similar to OECD TG 407 with regard to number of animals, duration of exposure and parameters investigated. Additionally, the hepatotoxic potential of the test item was investigated; microsomal preparations of the livers of all animals were examined to determine the total and specific cytochrome P450 content and UDPGT isoenzyme profile.

Study	Exploratory 28-day toxicity study in the rat by dietary administration.
Reference	[redacted] 2017
Test facility	[redacted]
Report reference	SA 11308
Guideline(s)	None
Deviations from the guideline	N/A
GLP	No
Test material	Isoflucypram, Batch: NLL 8674-19-4, Purity 98.6% w/w, CAS No 1255734-28-1.
Study acceptable	Yes, as a range-finding study. Lack of GLP is not considered to compromise the validity of the study as it is only a range-finding investigation.

Methods

Isoflucypram was administered continuously to Wistar rats (five/sex/group) for 28 days, at dietary concentrations of 0, 300, 1000 and 3000 ppm; these concentration equated to calculated mean intakes of 0, 22.8, 83.3 and 240 mg/kg bw/d in males and 0, 25.6, 86.5 and 285 mg/kg bw/d in females. The stability and homogeneity of the test

[1] https://webgate.ec.europa.eu/s-circabc/d/a/workspace/SpacesStore/83e2fb72-5d1f-4ada-be15-2246109e65d4/Interpretation%20of%20liver%20effects.pdf

item was demonstrated. A fully validated method of analysis for isoflucypram in the diet at the different concentrations tested is available (see document CA_B5 for further details).

Results

There were no treatment-related deaths or clinical signs of toxicity. Mean body weights of the high-dose male animals were at least 10% lower than controls at all time-points, reaching a maximum difference of 14.7% on day 22. This corresponded to an overall body-weight gain for these males that was 31.2% lower than their respective controls. Isoflucypram did not affect the body-weight development of males at the mid- or low-doses, or of females at any dose (see table below). Mean food consumption was not affected by treatment with isoflucypram in male or female rats at any dose. Overall, the RMS considers the changes in body weight and body-weight gain of high-dose male rats to be treatment-related and adverse.

Table B.6.3-1: Body weight development in the 28-day rat study with Isoflucypram

	Males				**Females**			
Dose level (ppm)	**0**	**300**	**1000**	**3000**	**0**	**300**	**1000**	**3000**
Body weight (g)								
Day 0	254.4	254.6	255.1	254.8	175.6	176.8	174.9	177.4
Day 29	402.7	418.4	420.1	**356.8****	223.4	223.1	224.6	226.5
% difference from control	-	-	-	**-11.4%**	-	-	-	-
Overall body-weight gain (g)	148.4	163.8	147.0	**102.1****	47.8	46.3	49.7	49
% difference from control	-	+10.4%	-	**-31.2%**	-	-	-	-

Statistically significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

Statistically significant changes to haematology parameters were observed in high-dose males only; lymphocyte and white blood cell counts were decreased by 37.6% and 33.8% respectively, whilst erythrocyte counts were slightly increased by 6.8% in comparison with control values. Changes to these parameters were small, did not show a clear dose-response, were not seen in females and were not repeated in any other studies, either with rats or with any other species; therefore, the RMS does not consider these haematology findings to be related to treatment with isoflucypram.

Small but statistically significant changes in clinical-chemistry measurements were noted in male rats at the high-dose; cholesterol, total protein and albumin concentrations were increased in comparison with controls by 26.5, 6.0 and 8.2% respectively. These are considered treatment-related and potentially adverse. Mean total bilirubin was statistically significantly lower in all dose groups, with evidence of a dose response (up to a maximum of 64.1/82% in males/females). Although related to treatment with isoflucypram, the RMS does not consider this to be an adverse effect because a *reduction* in bilirubin is not toxicologically significant. Furthermore, liver enzyme activity analysis (see table B.6.3.8) has revealed a marked induction of the UDPGT-bilirubin enzyme; this enzyme is responsible for the conjugation of bilirubin in the liver prior to its removal via the intestines. Therefore, the decrease in total bilirubin observed is likely to be a consequence of increased removal, secondary to liver enzyme induction. The observed changes to haematology and clinical chemistry parameters are summarised in table B.6.3.2 below.

Overall, adverse, treatment-related effects in some clinical-chemistry parameters were seen in males at the top dose.

Table B.6.3-2: Selected haematology and clinical-chemistry parameters in the 28-day rat study with isoflucypram

	Males				**Females**			
Dose-levels (ppm)	**0**	**300**	**1000**	**3000**	**0**	**300**	**1000**	**3000**
Haematology								
RBC (1012/L)	8.962	8.648	8.992	**9.576***	8.838	9.080	8.970	8.970
WBC (109/L)	12.81	13.84	10.89	**8.48***	8.62	9.48	7.58	8.51
Lymphocytes (109/L)	10.71	11.06	8.86	**6.68****	7.04	7.74	5.99	6.81
Clinical-chemistry								
Chol (mmol/L)	1.686	1.833	1.520	**2.132***	1.886	2	2.365	2.396
T. protein (g/L)	63.0	64.0	63.6	**66.8****	60.0	61.8	**67.3****	63.2
Albumin (g/L)	39.2	40.5	40.2	**42.4****	39.0	39.2	42.8	39.6

Dose-levels (ppm)	Males				Females			
	0	**300**	**1000**	**3000**	**0**	**300**	**1000**	**3000**
Bilirubin (µmol/L)	0.78	0.63	**0.26***	**0.28***	1.22	**0.68***	**0.30***	**0.22****
% difference from controls		19.2%	**66.6%**	**64.1%**		**44.3%**	**75.4%**	**82%**

Statistically significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

At 3000 ppm absolute liver weights were greater than controls by 13% in males (not statistically significant) and 64% in females, whilst relative liver weights at this dose were 31% greater in males and 63% greater in females. At the mid-dose of 1000 ppm, relative liver weights were increased by 14% and 30% in males and females respectively; absolute liver weights were also greater than controls at this dose but the change was statistically significant in females only (27%). Liver weights of both sexes at the low-dose were similar to controls. Overall, the liver weight changes seen (absolute and relative) were considered by the RMS to be related to treatment. In the mid-dose males, there were no associated histopathological findings (aside from hepatocellular hypertrophy, which is a morphological description related to increased functional capacity of the hepatocytes and not an indication of adversity), nor were there any clinical-chemistry findings indicative of specific liver toxicity. Therefore, the RMS considers that the relative liver weight increase of 14% in males at this dose is an adaptive and not an adverse response. The increases in relative liver weight in males at 3000 ppm and the absolute and relative weights in females from 1000 ppm, however, are considered to be adverse.

Relative thyroid weights were statistically significantly increased in males at 3000 ppm by 41% (a 21% increase in absolute thyroid weights in males at this dose and a 22% increase in absolute and relative weights in high-dose females were not of statistical significance). Changes in thyroid weights at the lower doses (also not statistically significant) were not related to treatment with isoflucypram, owing to the lack of a dose-response and the small magnitude of the changes. The relative thyroid weight increases seen in the top-dose males are considered by the RMS to be treatment-related. There were no treatment-related effects on the weights of any other organs. The table below summarises the observed changes in absolute and relative organ weights.

Overall, adverse (> 15%) increases in liver weight were seen from the mid-dose and adverse increases in thyroid weight were observed at the top dose.

Table B.6.3-3: Organ weight changes in the 28-day rat study with isoflucypram

Dose-levels (ppm)	Males				Females			
	0	**300**	**1000**	**3000**	**0**	**300**	**1000**	**3000**
Terminal body weight								
Weight (g)	381.6	393.3	374.1	**328.2****	208.4	208.2	207.9	210
% change	-	-	-	**14%****	-	-	-	-
Liver weights								
Absolute (g)	10.160	11.031	11.398	11.436	5.223	5.468	**6.629****	**8.562****
% change		9%	12%	13%		5%	**27%**	**64%****
Relative	2.663	2.800	**3.043***	**3.483****	2.510	2.634	**3.263****	**4.085****
% change		5%	**14%**	**31%**		5%	**30%**	**63%**
Thyroid weights								
Absolute (g)	0.01696	0.01818	0.01696	0.02050	0.01158	0.01303	0.01348	0.01416
% change		7%	0%	21%		13%	16%	22%
Relative	0.00443	0.00465	0.00455	**0.00623****	0.00555	0.00626	0.00648	0.00675
% change		5%	3%	**41%**		13%	17%	22%

Statistically significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

Upon necropsy, macroscopic changes were noted in the livers of several animals of both sexes at 3000 ppm, comprising enlarged and/or dark liver; prominent lobulation of the liver was also observed at this dose, but was confined to females. At the mid-dose, some male and female animals presented with an enlarged liver, whilst dark discolouration of the organ was noted in females only (see table below). There were no treatment related macroscopic findings in any other organs (at any dose) nor in the liver at 300 ppm.

Table B.6.3-4: Selected macroscopic findings (liver) in the 28-day rat study with isoflucypram

	Males (n=-5)				Females (n=5)			
Dose level (ppm)	**0**	**300**	**1000**	**3000**	**0**	**300**	**1000**	**3000**
Enlarged	0	1	2	2	0	0	1	5
Dark	0	0	0	3	0	0	3	2
Prominent lobulation	0	0	0	0	0	0	0	3

Microscopic findings were noted in the liver, kidneys and thyroid gland. Microscopic changes in the liver (see table below) comprised panlobular hepatocellular hypertrophy which was diffuse in nature. A total of 4/5 females (3 minimal, 1 slight) and 3/5 males (minimal) presented with this finding at 3000 ppm, whilst at 1000 ppm the finding was noted in 1/5 females (minimal). Overall, liver hypertrophy was seen in both sexes at the top dose and in females at the mid-dose.

Table B.6.3-5: Hepatocellular hypertrophy in the 28-day rat study with isoflucypram

	Males (n = 5)				Females (n = 5)			
Dose level (ppm)	**0**	**300**	**1000**	**3000**	**0**	**300**	**1000**	**3000**
Minimal	0	0	0	3	0	0	1	3
Slight	0	0	0	0	0	0	0	1
Total	0	0	0	3	0	0	1	4

In the thyroid, minimal to slight follicular cell hypertrophy (diffuse) was noted in male and female rats at the top- and mid-doses and in males at the low-dose (see table below). In males at the top-dose, but not females, this histopathological finding was accompanied by a statistically significant increase in the weight of the thyroid. At the low- and mid-dose, the incidence of follicular hypertrophy is very low and the finding is not associated with increased organ weight. Therefore, the histopathological changes in the thyroid observed at the low and mid dose are not considered adverse.

Table B.6.3-6: Thyroid follicular cell hypertrophy in the 28-day rat study with isoflucypram

	Males (n = 5)				Females (n = 5)			
Dose level (ppm)	**0**	**300**	**1000**	**3000**	**0**	**300**	**1000**	**3000**
Minimal	0	0	1	0	0	0	1	3
Slight	0	1	0	2	0	0	0	0
Total	0	1	1	2	0	0	1	3

In the kidneys a dose-related increase in tubular hyaline droplets was noted in males at all doses, and bilateral basophilic tubules were observed in males at 3000 and 1000 ppm. No treatment-related microscopic findings were observed in the female kidneys at any dose; any isolated incidences that were observed did not follow a clear dose-response and were of minimal severity only (see table below). The nature of the hyaline droplets found in male rats was confirmed by immunohistochemistry to be small α2u-globulin positive droplets, indicating an accumulation of α-2urinary-globulin in the renal proximal tubules. This is a common finding in the kidneys of male rats and is not relevant to humans, who do not secrete this protein. Overall, therefore, these kidney findings in male rats observed from the lowest dose of 300 ppm (22.8 mg/kg bw/d) are not relevant to humans.

Table B.6.3-7: Selected microscopic findings (kidneys) in the 28-day rat study with isoflucypram

	Males (n = 5)				Females (n = 5)			
	0	**300**	**1000**	**3000**	**0**	**300**	**1000**	**3000**
Basophilic tubules: bilateral								
Minimal	0	0	2	3	0	1	1	0
Slight	0	0	1	0	0	0	0	0
Total	0	0	3	3	0	1	1	0
Increased tubular hyaline droplets: proximal tubules								
Minimal	0	2	2	1	0	1	0	0
Slight	0	1	3	1	0	0	0	0
Moderate	0	0	0	3	0	0	0	0
Total	0	3	5	5	0	1	0	0

The effects of isoflucypram on the liver were investigated further at final necropsy using homogenised microsomal preparations, prepared from the remaining liver tissue of all animals. Total cytochrome P450 activity was determined by spectrophotometry (with a single quantification being performed for each sample), and was moderately increased in a dose-related manner in males at 3000 ppm and 1000 ppm; there were no statistically-significant effects on total cytochrome P450 activity at the low-dose in males, or in females at any dose (see table below). At the mid-and high-dose in males the most marked increases in enzyme activity were observed in relation to PROD, BROD, UDPGT-4 nitrophenol and UDPGT-bilirubin, whilst only moderate increases were noted in EROD and UDPGT-T4 activity. In females at these doses marked increases were seen in BROD, UDPGT-4 nitrophenol and UDPGT-T4, along with a moderate increase in PROD activity. The results of a positive control study (included in the table below), conducted with phenobarbital (75 mg/kg bw/d), β-naphthoflavone (75 mg/kg bw/d) and clofibrate (250 mg/kg bw/d) for 28-days' dietary exposure, revealed that the enzyme induction profile of isoflucypram most closely resembled that of the well characterised constitutive androstane receptor (CAR) activator phenobarbital. This provides evidence that isoflucypram is an activator of the constitutive androstane receptor (CAR) and/or pregnane X receptor (PXR). Further mechanistic studies investigating CAR/PXR activation have been submitted by the applicant and are presented in section B.6.8.2.

Overall, liver enzyme induction was seen in both sexes from 1000 ppm (83.2/86.5 mg/kg bw/d in M/F).

Table B.6.3-8: Liver enzyme activity (% of control values) in male and female rats administered isoflucypram.

	Isoflucypram, dietary ppm			**Positive control data**		
	Males					
Dose levels (ppm)	**300**	**1000**	**3000**	**PB**	**ßNF**	**CLO**
Total cytochrome P450	101	**124***	**135****	**205****	**147****	**139****
EROD	105	**136***	**157****	151	**717****	92
PROD	85	**161****	**515****	**1928****	209	230
BROD	129	**256****	**734****	**4291*****	**411****	**567****
UDPGT-4-nitrophenol	122	**214****	**247****	**226****	**300****	52
UDPGT-bilirubin	111	**244****	**317****	Not tested		
UDPGT-T4	102	**147***	**162****	Not tested		
	Females					
Dose levels (ppm)	**300**	**1000**	**3000**	**PB**	**ßNF**	**CLO**
Total cytochrome P450	86	89	92	**181****	**132****	109
EROD	100	112	128	132	**905****	90
PROD	116	140	143	**1249****	259	88
BROD	124	**229****	**524****	**5491****	**857****	**400****
UDPGT-4-nitrophenol	107	**198****	**304****	**202****	**512****	116
UDPGT-bilirubin	167	**250****	**401****	Not tested		
UDPGT-T4	135	**222****	**319****	Not tested		

Statistically significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

Discussion and conclusion

In conclusion, dietary administration of isoflucypram for 28-days resulted in lower body weights of male rats at the top dose of 240 mg/kg bw/d throughout the study, culminating in a lower overall body-weight gain value for these animals. Some indications of a minor impairment of liver function were reported at this dose in males and from the mid-dose (86.5 mg/kg bw/d) in females, comprising adverse (>15%) increased liver weights and hepatocellular hypertrophy (minimal in males and minimal to slight in females). In addition, minor alterations in clinical chemistry parameters (total protein, albumin and cholesterol) were seen at the top dose in males. In the thyroid, follicular cell hypertrophy was reported from the top dose of 240 mg/kg bw/d in males and from the mid-dose (86.5 mg/kg bw/d) in females; however, the weight of the thyroid was only statistically significantly increased in high-dose males (41%; relative). Therefore, thyroid effects are considered adverse only at the top dose. Liver enzyme induction investigations have shown that isoflucypram is a potent inducer of cytochrome P450 3A and the phase II enzyme UDPGT and a moderate inducer of cytochrome P450 2B from the mid dose of 83 mg/kg bw/d; similarities with the induction profile of phenobarbital suggest that CAR and/or PXR activation is probable, which is likely to be responsible for the treatment-related effects observed in the liver and thyroid. The kidney effects observed in males from the lowest tested dose of 300 ppm (22.8 mg/kg bw/d) were due to α2u-globulin accumulation and hence not relevant to humans.

A NOAEL of 300 ppm in females (equivalent to 25.6 mg/kg bw/d) is proposed by the RMS since no adverse effects were seen at this dose. A LOAEL of 1000 ppm (86.5 mg/kg bw/d) is proposed based on increased relative liver weights (30%).

The NOAEL proposed by the RMS for males is 1000 ppm (equivalent to 83.3 mg/kg bw/d) as no adverse effects were seen at this dose. A LOAEL of 3000 ppm (240 mg/kg bw/d) is proposed based on increased relative liver weights (by 31%), whilst relative thyroid weights were increased by 41%. Body-weight gain and final body weight were reduced at the LOAEL in males by 32% and 11.4% respectively. Therefore, the overall NOAEL from this 28-day study is 300 ppm (25.6 mg/kg bw/d). A LOAEL of 22.8 mg/kg bw/d was identified for kidney toxicity in male rats. This LOAEL is not relevant to the human risk assessment, but could be relevant to the wildlife mammal risk assessment.

(*[redacted] 2017)*

B.6.3.1.2 Oral 28-day study in mice

A 28-day dietary toxicity study has been conducted in mice. This dose-range finding study did not follow any particular guideline but was similar in design to the OECD TG 407.

Study	BCS-CN88460 – preliminary 28-day toxicity study in the mouse by dietary administration
Reference	[redacted] 2012
Test facility	[redacted]
Report reference	SA 11309
Guideline(s)	None
Deviations from the guideline	N/A
GLP	No
Test material	Isoflucypram. Batch: NLL 8674-19-4, Purity 98.6% w/w.
Study acceptable	Yes, as a range-finding study. Lack of GLP is not considered to compromise the validity of the study as it is only a range-finding investigation.

Methods

Groups of 5/sex C57BL/6J mice were administered isoflucypram in the diet for 28-consecutive days. Dietary concentrations of 0, 200, 800 and 2000 ppm equated to calculated mean intakes of 0, 32, 133 and 330 mg/kg bw/d in males and 0, 41, 149 and 374 mg/kg bw/d in females. The stability of the test item in the diet was reportedly demonstrated; however, it is not specified whether the homogenicity of the test item was assessed. A fully validated method of analysis for isoflucypram in the diet at the different concentrations tested is available (see document CA_B5 for further details).

Results

There were no treatment related deaths or clinical signs of toxicity. As summarised in the table below, isoflucypram did not have a marked effect on the body-weight development of male or female mice at any dose. The cumulative body-weight gain of high-dose female mice was, however, decreased by 69% over study days 1 to 8 (not statistically significant) and was accompanied by an initial reduction in food consumption (no further effects on food consumption were noted). These effects on body-weight gain were considered treatment-related and adverse.

Table B.6.3-9: Body weight development in the 28-day mouse study with isoflucypram

Dose levels ppm)		**Males**				**Females**			
	Day	**0**	**200**	**800**	**2000**	**0**	**200**	**800**	**2000**
Body weight (g)	1	22.10	22.32	22.38	21.90	17.62	17.26	17.48	17.84
	8	22.70	23.24	23.28	22.66	18.78	18.40	18.32	18.20
	15	23.30	23.76	24.32	23.50	19.28	19.10	19.04	19.72
	22	23.44	24.36	24.26	23.48	19.44	19.72	19.40	19.84
	29	24.32	25.08	25.30	24.76	20.40	20.30	20.40	21.00
Body-weight gain (g)	1-29	2.22	2.76	2.92	2.86	2.78	3.04	2.92	3.16

Statistically significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

Haematological parameters were not investigated in this study, but analysis of clinical-chemistry revealed statistically significant increases (relative to control values) in several parameters indicative of a slight impairment of liver function. At the high-dose of 2000 ppm ASAT was increased in males by 119%, whilst ALAT was increased by 110% in females and by over 4-fold in males; an increase in ALP, albeit less marked, was also observed in males at this dose (19%). Levels of these liver enzymes were also slightly increased in both sexes at the mid- and low-doses, but without statistical significance. Therefore, they are not considered to be of toxicological significance at the low and mid doses. Bilirubin was decreased in all females in a dose-dependent manner. This is consistent with the effects observed in the 28-day study with rats; however, in rats the effect was also observed in males. As previously discussed, although related to treatment with isoflucypram, this is not considered by the RMS to be an adverse effect, and is likely to be a consequence of increased bilirubin removal owing to the up-regulation of the UDPGT-bilirubin enzyme. Table B.6.3.10 below summarises the main clinical-chemistry findings.

Overall, adverse effects in some clinical-chemistry parameters, indicative of liver toxicity were seen at the top dose.

Table B.6.3-10: Clinical-chemistry changes in the 28-day mouse study with isoflucypram

	Males				Females			
Dose levels (ppm)	**0**	**200**	**800**	**2000**	**0**	**200**	**800**	**2000**
Bilirubin (µmol/L)	0.62	0.84	0.64	0.56	1.10	**0.62***	**0.46****	**0.52****
ASAT (IU/L)	89.8	86.2	133.2	**196.8***	108.6	125.0	109.2	148.2
ALAT (IU/L)	34.5	32.0	42.4	**159.0***	35.6	36.2	37.4	**74.8***
AP (IU/L)	110.6	102.4	125.0	**131.4***	178.6	181.4	181.0	183.2

Statistically significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

Absolute liver weights were statistically significantly increased at 2000 ppm in both sexes to an extent of 20.2% in males and 29.7% in females. Relative liver weights were also increased at this dose by 19% and 25% in males and females respectively. Increases at 800 ppm in absolute (11.3%) and relative (9.7%) liver weights in female mice were not statistically significant (see table below). The RMS considers the liver weight increases in males and females at the top-dose to be treatment related and adverse, owing to the magnitude of the increase, associated clinical chemistry changes (increases in ALP, ALAT and ASAT) and associated histopathological findings. There were no effects on the thyroid.

Table B.6.3-11: Liver weight changes in the 28-day mouse study with isoflucypram

	Males				Females			
Dose levels (ppm)	**0**	**200**	**800**	**2000**	**0**	**200**	**800**	**2000**
Liver weights								
Absolute (g)	0.893	0.906	0.965	**1.073****	0.723	0.761	0.805	**0.938****
% change		1.5%	8%	**20.2%**		5.3%	11.3%	**29.7%**
Relative	4.472	4.413	4.721	**5.325****	4.475	4.698	4.908	**5.593****
% change		1.3%	5.6%	**19%**		5%	9.7%	**25%**

Statistically significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

In 3/5 high-dose females, the increase in liver weight was accompanied by a dark discolouration of the organ. Further incidences of this macroscopic finding in one male at 800 ppm and one female at 200 ppm are considered as incidental findings and not related to treatment with isoflucypram (there were no associated liver weight increases or microscopic findings).

Various microscopic findings were noted in the liver upon necropsy, comprising hepatocellular hypertrophy, hepatocellular necrotic foci and hepatocellular single cell necrosis (see table below). Whilst hepatocellular hypertrophy is suggestive of an adaptive response and is not an indication of adversity, the observed necrotic foci and single cell necrosis in males from 800 ppm and in females at 2000 ppm is considered by the RMS to be both treatment-related and adverse.

Table B.6.3-12: Selected microscopic findings in the 28-day mouse study with isoflucypram

	Males				Females			
Dose levels (ppm)	**0**	**200**	**800**	**2000**	**0**	**200**	**800**	**2000**
Liver, N examined	5	5	5	5	5	5	5	5
Hepatocellular hypertrophy								

	Males				Females			
Dose levels (ppm)	**0**	**200**	**800**	**2000**	**0**	**200**	**800**	**2000**
Liver, N examined	5	5	5	5	5	5	5	5
Minimal	0	0	0	3	0	0	0	4
Slight	0	0	0	2	0	0	0	0
Total	**0**	**0**	**0**	**5**	**0**	**0**	**0**	**4**
Hepatocellular necrotic focus(i)								
Minimal	0	0	0	5	0	0	0	2
Slight	0	0	1	0	0	0	0	3
Total	**0**	**0**	**1**	**5**	**0**	**0**	**0**	**5**
Hepatocellular single cell necrosis								
Minimal	0	0	2	0	0	0	0	3
Slight	0	0	0	5	0	0	0	1
Total	**0**	**0**	**2**	**5**	**0**	**0**	**0**	**4**

Discussion and conclusion

Aside from an initial reduction in body-weight gain in high-dose females (-69% on days 1 to 8), which was at least partly if not entirely associated with reduced food consumption during this period, dietary administration of isoflucypram for 28-days, did not have a marked effect on the body-weight development of mice. The liver was the only affected organ; at the high-dose of 330/374 mg/kg bw/d relative liver weights were increased in males/females by 19% and 25% respectively and certain liver enzymes (ASAT, ALAT and ALP) were also increased at this dose. In addition to the hepatocellular hypertrophy observed at the high-dose in both sexes, adverse liver histopathology, in the form of hepatocellular necrotic focus and single cell necrosis, was evident in males from 133 mg/kg bw/d and in females at 374 mg/kg bw/d.

Therefore, the RMS proposes a NOAEL of 200 ppm (equivalent to 32 mg/kg bw/d) for males as no adverse effects were seen at this dose, whereas histopathological findings indicative of liver damage (hepatocellular necrotic foci and single cell necrosis) were seen at the next dose of 800 ppm (133 mg/kg bw/d) which is therefore the LOAEL.

A NOAEL of 800 ppm (equivalent to 149 mg/kg bw/d) for females is proposed by the RMS as no adverse effects were seen at this dose; adverse relative liver weight increases of 25% and incidences of necrotic foci and single cell necrosis were observed at 2000 ppm (374 mg/kg bw/d) which is the LOAEL. Therefore, the overall NOAEL from this 28-day study in mice is 32 mg/kg bw/d.

([redacted], *2012)*

B.6.3.1.3 Oral 28-day study in dogs

A non-guideline repeated-dose 28-day dietary toxicity study has been conducted in dogs. The study was intended as a dose-range finding study for the succeeding 90-day and one-year studies in dogs.

Study	BCS-CN88460 – preliminary 28-day toxicity study in the dog by dietary administration
Reference	[redacted]2014
Test facility	[redacted]
Report reference	SA 12107
Guideline(s)	None
Deviations from the guideline	N/A
GLP	No
Test material	Isoflucypram. Batch: NLL 8674-19-4, Purity 98.6% w/w, CAS No 1255734-28-1.
Study acceptable	Yes as a range-finding study. Lack of GLP is not considered to compromise the validity of the study as it is only a range-finding investigation.

Methods

Isoflucypram was administered to groups of two male and two female Beagle dogs at dietary concentrations of 0, 300, 1000 and 3000 ppm (estimated to be equivalent to 0, 12.7, 37.7 and 76.9 mg/kg bw/d and 0, 11.3, 36.5 and 90.2 mg/kg bw/d in males and females respectively) for 28-days. The stability and homogeneity of isoflucypram in the diet was determined prior to the the start of the study. Kinetic measurements of isoflucypram in blood were

also performed at the end of the study. A fully validated method of analysis for isoflucypram in the diet at the different concentrations tested is available (see document CA_B5 for further details).

Results

No animals died during the study; clinical signs thought to be treatment related were incidences of increased salivation on two occasions in one male at the top dose (days 15 and 22). The study authors have also reported a 'wasted appearance' for one top-dose female on day eight.

At the top-dose of 3000 ppm both of the male dogs and one female suffered body-weight losses of 0.2 to 0.5 kg (compared with gains of 0.6 to 1.2 kg in the control animals); the remaining female of the high-dose group generally maintained a static body-weight throughout the study. Isoflucypram did not affect the body weight of animals at the mid- or low-doses (see table below).

Table B.6.3-13: Body-weight development in the 28-day dog study with isoflucypram

Isoflucypram, ppm			Study Day					Body wt gain, kg
	Sex	Animal	1	8	15	22	29	1-29
0	M	0312	6.0	6.2	6.6	6.9	7.2	1.2
		0313	6.5	7.0	7.0	7.1	7.4	0.9
	F	0314	5.8	6.1	6.3	6.7	6.8	1.0
		0315	5.7	5.8	6.0	6.2	6.3	0.6
300	M	0316	6.9	7.1	7.4	7.5	7.9	1.0
		0317	6.1	6.5	6.6	6.8	7.0	0.9
	F	0318	5.8	6.1	5.9	6.1	6.4	0.6
		0319	5.6	5.8	5.8	6.2	6.0	0.4
1000	M	0320	6.3	6.4	6.5	6.5	6.7	0.4
		0321	7.8	8.0	8.3	8.5	8.7	0.9
	F	0322	5.8	5.8	5.8	6.1	6.2	0.4
		0323	6.0	6.2	6.2	6.4	6.4	0.4
3000	M	0324	6.6	6.4	6.2	6.1	6.1	-0.5
		0325	8.0	7.8	7.6	7.7	7.8	-0.2
	F	0326	5.8	5.7	5.8	5.8	5.9	0.1
		0327	5.6	5.3	5.1	5.2	5.3	-0.3

Body-weight losses at the top-dose were accompanied by a reduction in food intake for the three affected animals; food consumption for the remaining female in this dose-group was similar to her pre-treatment value, which was reflected in the slight body-weight gain observed in this animal. The body weight losses are considered to be treatment-related and adverse, even though they could have been the consequence of poor palatability.

Isoflucypram did not affect haematology or urinalysis parameters and there were no unusual ophthalmoscopic findings.

With regard to clinical chemistry (see table B.6.3.13 below), alkaline phosphatase (ALP) was increased in one male and both females (in comparison with their own pre-study value) at 3000 ppm and in one female at 1000 ppm. The applicant has reported that the lack of accompanying histopathological signs indicates that this was not an adverse finding. However, as increased ALP was also seen in the dog 90-d and 12-month studies, the RMS considers that the increase in ALP in these animals is related to treatment with isoflucypram and adverse. Total cholesterol concentration was decreased in one male and one female at the top dose; however, due to the large variability and when comparing the values with the pre-dose levels, it is most likely these were chance findings. It is also noted that effects on cholesterol were not seen in the dog 90-d and 12-month studies. Overall, there was an adverse increase in ALP at the top dose.

Table B.6.3-14: Selected clinical-chemistry findings in the 28-day dog study with isoflucypram

Isoflucypram, ppm			Alkaline phosphatase, IU/L		Cholesterol, mmol/L	
	Sex	Animal	Pre-study day 20	Study day 25	Pre-study day 20	Study Day 25
0	M	0312	110	119	2.79	2.75

Isoflucypram, ppm	Sex	Animal	Alkaline phosphatase, IU/L		Cholesterol, mmol/L	
			Pre-study day 20	Study day 25	Pre-study day 20	Study Day 25
		0313	130	117	3.75	3.34
	F	0314	129	123	2.91	2.51
		0315	97	115	2.68	2.66
300	M	0316	107	138	2.99	3.08
		0317	298	280	1.89	2.14
	F	0318	141	176	3.45	3.42
		0319	132	171	3.70	4.43
1000	M	0320	131	157	2.74	2.86
		0321	117	171	3.34	3.05
	F	0322	133	384	2.69	2.20
		0323	121	157	3.73	3.80
3000	M	0324	227	255	3.20	1.93
		0325	217	372	3.07	3.13
	F	0326	100	555	2.92	2.36
		0327	124	361	2.85	1.90

The plasma concentration of isoflucypram in treated animals was measured at the end of the study; measurements were taken pre-feeding and then 1, 2 and 4 hours after feeding. The largest values were generally found at the 4-hour post-feeding measurement, with pre-feeding values being at or below the Limit of Quantification (LOQ).

In females, absolute liver weights were increased by 19.6%, 35% and 28% whilst relative liver weights were increased by 24.7%, 39% and 52% at 300, 1000 and 3000 ppm respectively. In males at 3000 ppm absolute liver weights were increased by 26% and relative weights by 34.2%; absolute liver weights were also increased in males at the mid-dose (11.2%) but there was no corresponding increase in relative weights (see table below). The liver weight increases at 3000 ppm in males and from 300 ppm in females are related to treatment with isoflucypram and despite the lack of adverse histopathological changes are considered by the RMS to be of an adverse nature owing to the magnitude of the weight increases.

Table B.6.3-15: Liver weight changes in the 28-day dog study with isoflucypram

Dose levels (ppm)	Males				Females			
	0	**300**	**1000**	**3000**	**0**	**300**	**1000**	**3000**
Absolute liver weight (g)	254.3	263.3	282.9	320.5	191.2	228.7	257.9	244.7
% difference from controls		3.5%	11.2%	26%		19.6%	35%	28%
Relative liver weight (g)	3.56	3.55	3.68	4.78	2.96	3.69	4.12	4.50
% difference from controls		-	3.4%	34.2%		24.7%	39%	52%

Upon necropsy, an enlarged liver in one male at 3000 pm was the only macroscopic indication; microscopic findings were evident in both sexes (see table below) comprising hepatocellular hypertrophy (centrilobular to panlobular in distribution) at 3000 ppm and decreased severity of hepatocellular glycogen accumulation. Minimal accumulation of brown pigment in kupffer cells was noted in high-dose females only.

Table B.6.3-16: Microscopic liver findings in the 28-day dog study with isoflucypram

Dose levels (ppm)	Males				Females			
	0	**300**	**1000**	**3000**	**0**	**300**	**1000**	**3000**
Hepatocellular glycogen accumulation, diffuse								
Minimal	0	0	1	2	0	0	1	1
Slight	2	2	1	0	1	2	1	0
Moderate	0	0	0	0	1	0	0	0
Total	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**1**
Hepatocellular hypertrophy, centrilobular to panlobular								
Minimal	**0**	**0**	**0**	**2**	**0**	**0**	**0**	**1**
Accumulation of brown pigment in Kupffer cells								
Minimal	0	0	0	0	0	0	0	2

Discussion and conclusion

Treatment of male and female Beagle dogs with isoflucypram for 28-days, in a non-guideline study, resulted in body-weight losses or notably reduced body-weight gain at the top-dose of 76.9 mg/kg bw/d. Absolute and relative liver weights of females were greater than controls in all treated groups from 11.3 mg/kg bw/d; in males, this effect was noted at the top dose of 76.9 mg/kg bw/d (34.2%) only. In most cases, increased ALP accompanied the increased liver weights seen at the top dose and the only histopathological findings noted at necropsy were those indicative of an adaptive response rather than of adversity (hepatocellular hypertrophy); however, despite this lack of adverse histopathology, the absolute and relative liver weight increases seen at 76.9 mg/kg bw/d in males and from 11.3 mg/kg bw/d in females are considered by the RMS to be adverse, solely based on the magnitude of the weight increases. In addition, an adverse increase in ALP was seen at the top dose.

However, owing to the small number of animals used (2/sex/dose) the RMS does not consider it appropriate to set a robust NOAEL/LOAEL from this study.

([redacted] *2014)*

B.6.3.2. Oral 90- day studies

B.6.3.2.1. Oral 90-day study in rats

A 90-day dietary toxicity study, generally compliant with OECD guideline 408 has been conducted in rats.

Study	BCS-CN88460 90-day toxicity study in the rat by dietary administration
Reference	[redacted]2014
Test facility	[redacted]
Report reference	SA 12012
Guideline(s)	OECD 408
Deviations from the guideline	None
GLP	Yes
Test material	isoflucypram. Batch: NLL 8674-19-4, purity : 97.7% (w/w)
Study acceptable	Yes

Methods

Isoflucypram was administered to groups of 10 male and 10 female Wistar rats for 90-days at concentrations of 0, 100, 300 and 1000 pm. These dietary concentrations corresponded to calculated mean intakes of 0, 6.34, 18.4 and 63.5 mg/kg bw/d in males and 0, 7.92, 21.9 and 80.9 mg/kg bw/d in females. An additional 10 male and 10 female animals were added to the control- and high-dose groups in order to assess the reversibility of any noted effects. These recovery animals were maintained on control diet for a further month after the initial 90-day treatment period. A fully validated method of analysis for isoflucypram in the diet at the different concentrations tested is available (see document CA_B5 for further details).

Results

There were no treatment-related deaths or clinical signs of toxicity. No adverse findings were reported during the neurotoxicity assessment (functional observation battery and motor activity measurements) which was conducted during study week eleven. An ophthalmological examination conducted at the end of the study revealed no treatment-related abnormalities.

Isoflucypram did not have a marked effect on mean body weight or body-weight gain. At the high-dose of 1000 ppm, mean overall body-weight gain was reduced in males by 12% and in females by 8%, resulting in a mean body weight at the end of the treatment period that was lower in males only (-7%); however, none of these changes were of statistical significance and by the end of the recovery period overall body-weight gain in the high-dose males was statistically significantly greater than in the control group. The mean body weight and body-weight gain of treated females in the recovery group were similar to those seen in recovery controls, with the exception of a slightly higher body weight recorded at the end of the recovery period. The study authors attributed this to a body-weight loss in the control groups (owing to a problem with the water distribution system) and not to treatment with isoflucypram. There was no effect on food consumption at any dose. The table below summarises the body-weight development of animals in both the treatment and the recovery groups.

Overall, an adverse effect on body weight gain was seen at the top dose in males only.

Table B.6.3-17: Body-weight development in the 90-day rat study with isoflucypram

	Males	**Females**

Dose levels (ppm)	0	100	300	1000	0	100	300	1000
Main study phase								
Initial body wt, g	213.3	214.6	214.4	213.4	177.3	183.4	185.0	185.3*
Body wt, g, week 13	547.9	545.3	545.3	507.1	297.3	294.4	297.6	295.5
Body wt gain, g	334.6	330.7	330.9	293.6	120.0	111.0	112.6	110.1
Recovery phase								
Initial body wt, g	552.0			498.8	289.7			304.0
Body wt, g, day 29	573.1			536.5	285.0			310.4*
Body wt gain, g	21.1			37.7	- 4.7			6.4

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Blood samples were collected on the scheduled day of sacrifice (day 93, 94 or 95 of the main study phase and day 30 or 31 of the recovery phase). Haematological parameters were not affected by treatment with isoflucypram. With regard to clinical chemistry measurements, total bilirubin was statistically significantly reduced in males at 1000 ppm and in females at 1000 and 300 ppm; however, this trend did not persist into the four week recovery period, after which the total bilirubin values were similar across the control and high-dose groups. The observed decreases in bilirubin are likely to have resulted from the induction of the UDPGT-bilirubin enzyme (seen in the 28-day rat study) and the resulting greater bilirubin clearance. Cholesterol was slightly increased in high-dose females after 90-days' exposure; in recovery high-dose males and females cholesterol was slightly decreased (a decrease in cholesterol is not clinically significant). Clinical chemistry findings are summarised in the table below. Overall, only the increase in cholesterol at the top dose in females is considered adverse.

Table B.6.3-18: Selected clinical chemistry findings in the 90-day rat study with isoflucypram

Dose levels (ppm)	Males				Females			
	0	100	300	1000	0	100	300	1000
Main study phase								
Total bilirubin, µmol/L	0.94	0.97	0.96	0.56*	1.85	1.52	1.16*	0.68**
Total cholesterol, mmol/L	1.528	1.639	1.750	1.716	1.933	1.838	1.973	**2.302***
Recovery phase								
Total bilirubin, µmol/L	1.13			0.84	1.70			1.79
Total cholesterol, mmol/L	1.584			1.403*	1.889			1.638*

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Urinalysis revealed the presence of cellular casts in 5/10 high-dose males. This finding was associated with microscopic changes in the kidney and is related to treatment with isoflucypram (see histopathology below). No cellular casts were observed in the urine of high-dose recovery males.

Changes in absolute and relative liver and thyroid weights are shown in Table 6.3.2.3. In females the thyroid was not affected, but liver weights were increased by 20% (absolute) and 28% (relative). In males of this dose-group, thyroid weights were increased by 13% (absolute; not statistically significant) and 21% (relative), whilst relative liver weights were 11% greater than controls. None of these changes persisted to the end of the recovery period.

Overall, adverse increases in liver (> 15%) and thyroid weights were seen at the top dose.

Table B.6.3-19: Selected organ weights from the 90-day rat study with isoflucypram

Dose levels (ppm)	Males				Females			
	0	100	300	1000	0	100	300	1000
Liver weight (g)	11.24	11.27	11.17	11.67	6.207	6.240	6.342	**7.469****
% change								**20%**
Relative liver weight (g)	2.162	2.175	2.163	**2.397****	2.158	2.254	2.231	**2.754*****
% change				**11%**				**28%**
Thyroid weight (g)	0.0188	0.0200	0.0179	0.0213	0.0153	0.0144	0.0159	0.0150

% change				13%				
Relative thyroid weight (g)	0.00363	0.00386	0.00347	**0.00439***	0.00533	0.00520	0.00558	0.00553
% change				**21%**				

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Gross pathology examinations of the high-dose animals revealed an enlarged liver in 8/10 females and 2/10 males, with histopathological correlates evident in females. Two females at the lower doses (one at 300 ppm and one at 100 ppm), in addition to one male and two females of the treated recovery group, also presented with an enlarged liver; however, in these latter cases there were no associated microscopic findings. Therefore, although all potentially related to treatment with isoflucypram, only those findings in the high-dose group of the main study were at an advanced enough stage to be considered adverse.

Histopathological findings were seen in the liver, thyroid and kidney. Hepatocellular hypertrophy, which was periportal to panlobular in distribution and minimal to slight in severity, was noted in females at 1000 ppm; no findings of this nature were noted in any animals of the recovery group (see table below).

Table B.6.3-20: Selected microscopic findings (liver) in the 90-day rat study with isoflucypram.

Dose levels (ppm)	**Males**				**Females**			
	0	**100**	**300**	**1000**	**0**	**100**	**300**	**1000**
N examined	10	10	10	10	10	10	10	10
Hepatocellular hypertrophy: periportal to panlobular								
Minimal	0	0	0	0	0	0	0	**5**
Slight	0	0	0	0	0	0	0	**1**
Total	0	0	0	0	0	0	0	**6**

In high-dose males and females, microscopic findings were evident in the thyroid comprising minimal follicular cell hypertrophy and colloid alteration (see table below). These findings were not evident in the recovery group (in recovery males the severity but not the incidence of colloid alteration was increased in comparison with the respective controls).

Table B.6.-21: Selected microscopic findings (thyroid) in the 90-day rat study with isoflucypram

Dose levels (ppm)	**Males**				**Females**			
	0	**100**	**300**	**1000**	**0**	**100**	**300**	**1000**
N examined	10	10	10	10	10	10	10	10
Follicular cell hypertrophy: diffuse								
Minimal	1	0	1	5	0	0	0	5
Total	1	0	1	5	0	0	0	5
Colloid alteration								
Minimal	2	0	1	5	0	0	0	2
Total	2	0	1	5	0	0	0	2
Recovery phase								
N examined	10	-	-	10	10	-	-	10
Colloid alteration		-	-			-	-	
Minimal	2	-	-	6	0	-	-	1
Slight	1	-	-	0	0	-	-	0
Total	3	-	-	6	0	-	-	1

As described in table B.3.2.6 (below), proximal tubule hyaline droplets were observed in 10/10 high-dose males, associated in some cases with a higher severity of bilateral basophilic tubules (minimal granular casts were also present in some males at this dose). All of these findings persisted through to the end of the recovery period. Hyaline droplets were also present at the mid-and low-doses in 7/5 and 4/5 males respectively, but the applicant did not consider these to be treatment-related as the finding was also evident in 4/5 control males; however, the RMS considers that the findings at 1000 and 300 ppm are related to treatment with isoflucypram. The observed higher incidence and severity of hyaline droplets in the renal proximal tubules of treated male rats was correlated with an accumulation of α-2 urinary globulin (identified by immunohistochemistry). Small α-2 urinary globulin positive droplets were observed in control males and increased in severity in treated males in a dose-related

manner. Furthermore the size and shape of the droplets were changed from moderately sized in controls to large polyangular at 1000 ppm and the number of affected tubules also increased (see table 6.3.2.7 below). Therefore, it can be seen that dietary administration of isoflucypram increases the accumulation of α-2 urinary globulin in the kidney tubules of male rats. Such an effect (also observed in the 28-day rat study) is rat specific and is not relevant to humans, owing to the fact that this protein is not secreted in humans.

Overall, the kidney toxicity seen in male rats at the top dose is not relevant to humans.

Table B.6.3-22: Selected microscopic findings (kidneys) in the 90-day rat study with isoflucypram

	Males				Females			
Dose levels (ppm)	**0**	**100**	**300**	**1000**	**0**	**100**	**300**	**1000**
N examined	10	10	10	10	10	10	10	10
Hyaline droplets: proximal tubules								
Minimal	3	4	5	4	0	0	0	0
Slight	1	0	2	5	0	0	0	0
Moderate	0	0	0	1	0	0	0	0
Total	4	4	7	10	0	0	0	0
Basophilic tubules: bilateral								
Minimal	4	0	0	1	0	0	0	0
Slight	0	0	1	5	0	0	0	0
Total	4	0	1	6	0	0	0	0
Granular cast(s)								
Minimal	0	0	0	2	0	0	0	0
Total	0	0	0	2	0	0	0	0
Recovery phase								
N examined	10	-	-	10	10	-	-	10
Basophilic tubules: bilateral								
Minimal	2	-	-	4	0	-	-	1
Slight	0	-	-	1	0	-	-	0
Total	2	-	-	5	0	-	-	1
Granular cast(s)		-	-			-	-	
Minimal	0	-	-	3	0	-	-	0
Total	0	-	-	3	0	-	-	0

Table B.6.3-23: Alpha-2 urinary globulin accumulation profile in males of the 90-day rat study with isoflucypram

	Males			
Dose levels (ppm)	**0**	**100**	**300**	**1000**
N examined	10	10	10	10
Alpha2u-globulin immunohistochemical staining				
Minimal	6	7	5	2
Slight	4	3	4	6
Moderate	0	0	1	2
Total	10	10	10	10
Mean severity	1.40	1.30	1.60	2.00
Recovery phase				
N examined	9			10
Alpha2u-globulin immunohistochemical staining				
Minimal	7			7
Slight	2			3
Total	9			10
Mean severity	1.22			1.30

Discussion and conclusion

In a OECD guideline-compliant 90-day study, Wistar rats received isoflucypram in the diet at 100, 300 or 1000 ppm. High-dose and control satellite groups were kept for a further month after exposure. A decrease in body weight gain was seen in males at the top dose of 1000 ppm (63.5 mg/kg bw/d). At the high-dose of 63.5 and 80.9 mg/kg bw d (in males and females respectively) relative liver weights were increased in females by 28% and relative thyroid weights in males were increased by 21%; histopathological correlates were evident at this dose in these organs, comprising hepatocellular hypertrophy (liver) and follicular cell hypertrophy/colloid alteration (thyroid). There was also an increase in cholesterol levels in the top dose females. Urinalysis and microscopic findings in the kidneys of male rats at the high-dose were attributed to alpha-2-urinary-globulin accumulation and hence not considered as relevant to humans. There were no treatment-related findings at the mid- or low-doses.

The RMS's proposed NOAEL is therefore 300 ppm (equivalent to 18.4 mg/kg bw/d in males and 21.9 mg/kg bw/d in females) since no adverse effects were seen at this dose level. At the LOAEL of 63.5/80.9 mg/kg bw/d (top dose) in M/F, increases in relative thyroid and liver weights with associated histopathology were observed. There was also an increase in cholesterol in females.

([redacted] *2014)*

B.6.3.2.2. Oral 90-day study in mice

One 90-day dietary toxicity study generally compliant with OECD 408 has been conducted in mice.

Study	BCS-CN88460 90-day toxicity study in the mouse by dietary administration
Reference	[redacted] 2013
Test facility	[redacted]
Report reference	SA 12103
Guideline(s)	OECD 408 (1998)
Deviations from the guideline	None
GLP	Yes
Test material	isoflucypram, Batch : NLL8674-21-4
Study acceptable	Yes

Methods

Isoflucypram was added continuously to the diet of C57BL/6J mice (10/sex/group) for 90 days. Concentrations of 0, 100, 300 and 1000 ppm equated to calculated mean intakes of 0, 17, 51 and 168 mg/kg bw/d and 0, 20, 60 and 207 mg/kg bw/d in males and females respectively. A fully validated method of analysis for isoflucypram in the diet at the different concentrations tested is available (see document CA_B5 for further details).

Results

No animals died during the study and there were no treatment-related clinical signs of toxicity. Isoflucypram did not have any effect on the body-weight development of mice (see table below) and neither was there any effect on food consumption. Small reductions in body-weight gain in males at the mid- and low-doses compared with controls were considered not related to treatment as there was no clear dose-response relationship and overall body weights were similar across all groups.

Table B.6.3-24: Body weight development in the 90-day mouse study with isoflucypram

Dose levels (ppm)		**Males**				**Females**			
	Day	**0**	**100**	**300**	**1000**	**0**	**100**	**300**	**1000**
Body weight (g)	1	20.61	20.67	20.58	20.53	17.73	17.58	17.46	17.55
	29	21.83	21.69	21.86	21.62	20.76	20.52	20.45	20.28
	57	25.43	24.98	25.29	24.74	21.78	21.32	21.12	21.15
	92	26.44	25.69	26.42	25.25	22.40	21.92	21.70	22.10
Body-weight gain (g)	1-29	3.26	2.87	3.16	2.76	3.03	2.94	2.99	2.73
	1-57	4.82	4.31	4.71	4.21	4.05	3.74	3.66	3.60
	1-92	5.83	5.02	5.84	4.72*	4.67	4.34	4.24	4.55

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Clinical chemistry analysis revealed alterations in the serum concentrations of bilirubin, albumin and cholesterol (see table below). A statistically significant decrease in total bilirubin concentration was evident in both sexes at

1000 ppm, which was treatment-related but not toxicologically significant; a decrease in bilirubin concentration in females at 100 ppm was attributed to low values from two haemolysed samples and not to treatment with isoflucypram. Albumin values were increased and and cholesterol values decreased in high-dose males; although statistically significant, the changes were not accredited to treatment with isoflucypram because of the low magnitude of the changes (albumin) and the fact that all individual values were within the control range (cholesterol).

Overall, there were no treatment-related, adverse effects in clinical-chemistry parameters.

Table B.6.3-25: Clinical chemistry alterations in the 90-day mouse study

Dose levels (ppm)	Males				Females			
	0	**100**	**300**	**1000**	**0**	**100**	**300**	**1000**
Total bilirubin, umol/L	1.23	0.88	0.88	0.71**	1.24	0.71**	0.92	0.84*
Total cholesterol, mmol/L	1.892	2.001	1.809	1.570*	1.564	1.547	1.576	1.577
Albumin, g/L	35.8	35.1	35.0	33.9**	35.6	35.9	35.8	35.7

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Absolute and relative liver weights of the high-dose males were increased in comparison with controls by 11.6% and 17.8% respectively. In females at this dose, liver weights were increased by 12.6% (absolute) and 16.8% (relative). Histopathological evaluation of this organ revealed alterations to the pattern of vacuolation at the top dose which is likely to have arisen as a result of an increase in xenobiotic metabolising enzymes in the liver; specifically, a loss of diffuse hepatocellular vacuolation and of vacuolation in the periportal area of the hepatic lobules. A greater incidence of centrilobular hepatocellular vacuolation was also reported at the top dose. In addition, a higher incidence of micronucleated hepatocytes was seen in males from the mid dose (0, 0, 2, 2). This finding was also reported in the mouse carcinogencity study in males treated with the top dose of 1250 ppm (but not at 250 ppm), in association with other histopathological effects. The RMS is of the view that the effect is treatment-related, but that at the mid dose of 300 ppm, in the absence of other liver findings (including organ weight changes), it should not be considered adverse. The table below summarises the organ weight changes and histopathology findings in the liver.

Overall, adverse (>15%) increases in liver weight were seen at the top dose in both sexes.

Table B.6.3-26: Liver weight changes and histopathological findings in the 90-day mouse study with isoflucypram

Dose levels (ppm)	Males				Females			
	0	**100**	**300**	**1000**	**0**	**100**	**300**	**1000**
Liver weight (g)	0.912	0.918	0.989	**1.018***	0.791	0.818	0.794	0.891*
% difference from controls	-	-	8.4%	**17.8%**	-	-	-	11.6%
Relative liver weight (g)	4.107	4.232	4.447*	**4.840****	4.183	4.518*	4.410	**4.886****
% difference from controls	-	-	8.3%	**17.8%**	-	8%*	5.4%	**16.8%**
Hepatocellular vacuolation, mainly centrilobular, diffuse								
Minimal	1	0	0	3	1	0	3	4
Slight	0	0	0	0	1	1	1	4
Total	1	0	0	3	2	1	4	8
Hepatocellular vacuolation, diffuse								
Minimal	1	5	1	2	2	1	3	0
Slight	8	5	9	5	6	8	3	1
Total	9	10	10	7	8	9	6	1
Multinucleated hepatocytes	0	0	2	2	0	0	0	0

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Discussion and conclusion

Treatment of mice with isoflucypram in the diet at 100, 300 or 1000 ppm for 90-days, in a standard OECD 408 guideline study, resulted in an increase in relative liver weights in high-dose males and females (168 and 207 mg/kg bw/d respectively). In males, liver weights (relative to body weight) were 17.8% greater than controls, whilst in females relative liver weights were 16.8% greater. These increases were associated with histopathological findings (vacuolation). A NOAEL of 300 ppm (equivalent to 51 mg/kg bw/d in males and 60 mg/kg bw/d in females) is therefore proposed by the RMS as no effects were seen at this level of dietary exposure.

([redacted] 2013)

B.6.3.2.3. Oral 90-day study in dogs

The short-term oral toxicity of isoflucypram has been investigated in dogs after 90-days' repeated exposure via the diet, according to OECD TG 409.

Study	BCS-CN88460 90-day toxicity study in the dog by dietary administration
Reference	[redacted] 2015
Test facility	[redacted]
Report reference	SA 13272
Guideline(s)	OECD TG 409 (1998)
Deviations from the guideline	None
GLP	Yes
Test material	isoflucypram, Batch : 2013-006492, Purity 94.2% w/w
Study acceptable	Yes

Methods

Isoflucypram was administered in the diet of Beagle dogs (4/sex/dose) for 90 days at dietary concentrations of 0, 170, 500 and 1500 ppm, which equated to calculated mean intakes of 0, 5.5, 15.9 and 54.0 mg/kg bw/d in males and 0, 5.5, 16.2 and 54 mg/kg bw/d in females. A fully validated method of analysis for isoflucypram in the diet at the different concentrations tested is available (see document CA_B5 for further details).

Results

No animals died during the study and the only clinical observation was an increase in salivation in 2/4 females at 1500 ppm. At the high-dose of 1500 ppm, mean body-weights were reduced during the second half of the study only, by 7% in males and 8% in females, resulting in an overall mean body-weight gain that was 39% lower than controls in both sexes; no effects on body-weight development were noted at the mid- or low-doses (see table below). There was no effect on food consumption at any dose in either males or females.

Table B.6.3-27: Body-weight development in the 90-day dog study with isoflucypram

Dose levels (ppm)	Day	Males				Females			
		0	**170**	**500**	**1500**	**0**	**170**	**500**	**1500**
Body weight (kg)	1	7.03	7.00	7.15	7.20	6.63	6.53	6.73	6.65
	8	7.35	7.25	7.53	7.45	6.80	6.88	6.85	6.80
	92	9.00	8.48	8.73	8.40	8.35	8.03	8.13	7.70
Body weight gain (kg)	1-8	0.33	0.25	0.38	0.25	0.18	0.35	0.13	0.15
	1-92	1.98	1.48	1.58	**1.20****	1.73	1.50	1.40	1.05

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Urinalysis and haematological parameters were not affected by treatment with isoflucypram and the only treatment-related changes to clinical chemistry parameters were consistent with previous studies, in which the liver was the primary target organ of isoflucypram (see table B.6.3.2.12 below). Clinical chemistry measurements revealed an increase in ALP (in comparison with controls) in males and females at 1500 ppm (139% and 174% respectively at the end of the study) and a decrease in bilirubin was observed in males and females (although statistical significance was only achieved in males at 1500 and 500 ppm). There was also a slight increase (by 59-66%) in ALP at 500 ppm. Given the magnitude of the effect, the increase in ALP at 500 ppm in isolation is not considered adverse; however, owing to the clear dose-reponse observed across the dose-range, it is considered to be related to treatment with isoflucypram. It is also noted that at this dose level there were no toxicologically significant increases in liver weight and no associated liver hypertrophy. The effect on bilirubin levels, although treatment related, is not toxicologically significant because a decrease in this parameter is not of clinical relevance

and is likely to be related to increased bilirubin clearance, owing to liver enzyme induction. Therefore, adverse increases in ALP were seen only at the top dose.

Table B.6.3-28: Clinical chemistry findings from the 90-day dog study with isoflucypram

Dose levels (ppm)		Males				Females			
	Week	**0**	**170**	**500**	**1500**	**0**	**170**	**500**	**1500**
Alkaline phosphatase IU/L	PS	84.0	86.3	87.5	93.0	70.8	76.0	118.0	123.5
	7	104.5	136.5	139.8	217.5*	103.3	122.3	176.3	291.3*
	12-13	89.3	135.5	142.3	**213.5***	103.5	128.0	171.8	**284.0***
% difference		-	51%	59%	**139%**	-	23.7%	66%	**174%**
Total bilirubin µmol/L	PS	0.65	0.88	0.53	0.33	0.68	0.93	0.73	1.03
	7	0.78	0.90	0.40	0.45	0.73	0.33	0.68	0.48
	12-13	0.75	0.70	**0.33***	**0.30***	0.73	0.33	0.40	0.33
% difference		-	-7%	**-56%**	**-60%**				

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

In high-dose males, relative liver weights were statistically significantly increased in comparison with controls by 42.8%. Although not reaching statistical significance, absolute liver weight in top-dose males and relative weight in top-dose females were also noticeably increased by 34% and 20% above the control value respectively (see table B.6.3.2.13 below). There were no treatment-related effects on liver weights at the mid- or low-doses and no other organs were affected.

Table B.6.3-29: Liver weight changes in the 90-day dog study with isoflucypram

Dose levels (ppm)	Males				Females			
	0	**170**	**500**	**1500**	**0**	**170**	**500**	**1500**
Liver wt (g)	252.2	269.4	284.0	**338.5**	279.4	278.3	291.1	308.1
% change				**34%**				10%
Relative liver weight (g)	2.83	3.16	3.28	**4.04****	3.34	3.48	3.60	**4.03**
% change				**42.8%**				**20%**

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

The increased relative liver weights in high-dose males were associated with histopathological changes in the liver, comprising centrilobular hepatocellular hypertrophy (3/4, minimal); in two of these animals this was further associated with eosinophilic intracytoplasmic vacuoles. There were no other findings upon necropsy and ophthalmological investigations revealed no unusual results. The table below summarises the histopathological changes observed in the liver.

Overall, liver histopathology was seen at the top dose in males.

Table B.6.3-30: Histopathological observations in the 90-day dog study with isoflucypram

Dose levels (ppm)	Males				Females			
	0	**170**	**500**	**1500**	**0**	**170**	**500**	**1500**
Hepatocellular hypertrophy, centrilobular								
Minimal	0	0	0	3	0	0	0	0
Slight	0	0	0	0	0	0	0	0
Total	0	0	0	3	0	0	0	0
Intracytoplasmic eosinophilic inclusion								
Total	0	0	0	2	0	0	0	0

Discussion and Conclusion

In a standard OECD guideline 409 study, administration of isoflucypram in the diet of dogs for 90-days at the top dose of 1500 ppm (50.4/55 mg/kg bw/d in M/F) resulted in salivation in males and in a lower (by 39%) body weight gain at the end of the study. In these high-dose males and females, mean relative liver weights were increased by 42.8% and 20% respectively in comparison with controls (the increase in females was not statistically significant); mean absolute liver weight, whilst not reaching statistical significance, was also notably increased in male dogs by 34% above the weight in controls. The changes in liver weights were accompanied in males by

minimal hepatocellular hypertrophy and intracytoplasmic eosinophilic inclusion. A statistically significant increase in ALP was also noted in males and females at this dose.

A NOAEL of 500 ppm (equivalent to 15.9 and 16.2 mg/kg bw/d in males and females respectively) is proposed by the RMS as at this dose no adverse effects were seen, with reductions in body-weight gain and liver weight increases (with associated clinical chemistry findings and histopathology), in males and females at the LOAEL/top dose of 1500 ppm (50.4 and 54 mg/kg bw/d respectively) which was the next lowest dose level.

([redacted] *2015)*

B.6.3.2.4. Oral 12-month study in dogs

Isoflucypram has been investigated in a 12-month OECD 452 guideline compliant study in Beagle dogs.

Study	BCS-CN88460 - Chronic toxicity study in the dog by dietary administration
Reference	[redacted] 2017
Test facility	[redacted]
Report reference	SA 14092
Guideline(s)	OECD TG 452 (2009)
Deviations from the guideline	None
GLP	Yes
Test material	isoflucypram, Batch : 2013-006492, Purity 94.2% w/w
Study acceptable	Yes

Methods

Dietary concentrations of 0, 150, 600 and 1800 ppm of isoflucypram were administered to Beagle dogs (4/sex/dose) for 12-months, corresponding to calculated mean intakes of 0, 4.2, 18.8 and 60.2 mg/kg bw/d in males and 0, 4.2, 17.6 and 49.8 mg/kg bw/d in females. A validated method of analysis for isoflucypram in the diet at the different concentrations tested is available (see document CA_B5 for further details). It is noted that the dose formulations were not verified on every occasion; however, as the findings and associated dose-responses from this study are consistent with those of the previous 90-day study in the dog, in which the dose formulations were verified on every occasion, it is concluded that the method of analysis of this 12-month study is acceptable.

Results

There were no treatment-related deaths; one female at 150 ppm was killed for humane reasons, but the poor clinical condition of the animal was attributed to a congenital anomaly. This animal was therefore excluded from the analyses. An increase in salivation in one male at 1800 ppm was the only clinical sign reported.

At the high-dose of 1800 ppm, during the first week of treatment, a mean body-weight loss was observed in females whilst in males, mean body-weight gain was reduced in comparison with controls. Mean cumulative body-weight gains were also reduced on several occasions in females of this dose-group over the remainder of the study (see table below). There were effects on body weight and body weight gain also at the mid dose, especially in males. The RMS concludes that there was an adverse effect on body weight and body weight gain at the top dose of 1800 ppm in both sexes and at the mid dose of 600 ppm in males; This was more pronounced during the first week of treatment.

Table B.6.3-31: Body weight development in the 12-month dog study with isoflucypram

Dose levels (ppm)		**Males**				**Females**			
	Day	**0**	**150**	**600**	**1800**	**0**	**150**	**600**	**1800**
Body weight (kg)	*1*	7.08	7.05	6.93	6.98	7.40	8.10	7.10	7.13
	9	7.23	7.25	7.05	7.00	7.45	8.17	7.23	7.00
	93	8.60	8.55	8.05	8.23	8.75	9.73	8.13	7.90
	184	9.15	8.90	8.45	8.60	9.30	10.30	8.73	8.40
	361	9.65	9.10	8.60	8.83	9.95	11.10	9.08	8.85
Body weight gain (kg)	*1-9*	0.019	0.025	0.016	0.003	0.05	0.07	0.13	-0.013
	1-93	1.53	1.50	1.13	1.25	1.35	1.63	1.03	0.78
	1-184	2.08	1.85	1.53	1.63	1.90	2.20	1.63	1.28
	3-361	2.58	2.05	1.68	1.85	2.55	3.00	1.98	1.73

At the top-dose (1800 ppm) in females, food consumption was reduced by 25% during the first week of the study, corresponding to the initial body-weight losses in these animals; overall food consumption (days 1 to 364) was reduced by 10% when compared with controls in this group. There were no effects on food consumption in females at the lower doses or in males at any dose.

No unusual haematology findings were noted but clinical-chemistry changes consistent with the liver as a target organ were noted at 1800 ppm in both sexes (see table below). ALP was increased by approximately 4.5-fold and 2-fold in males and females respectively at this dose, but also at 600 ppm in males; alterations to levels of bilirubin were also noted in both sexes, but were not toxicologically significant because, as in previous studies, the trend was for a reduction and not an increase in this parameter. Urinalysis did not reveal any treatment-related changes.

Overall, adverse increases in ALP were seen from the mid dose of 600 ppm.

Table B.6.3-32: Clinical chemistry findings in the 12-month dog study with isoflucypram

Dose levels (ppm)									
		Males				Females			
	Month	**0**	**150**	**600**	**1800**	**0**	**150**	**600**	**1800**
Alkaline phosphatase IU/L	*Pre-study*	89.8	98.0	132.3	137.3	116.0	114.0	96.3	82.0
	4	69.8	87.3	**156.5***	**266.0****	110.3	102.5	132.5	185.5
	6	63.0	85.0	127.3	**243.3****	105.0	83.0	144.5	176.3
	12	57.3	82.5	152.5	**268.8****	108.0	106.0	180.0	**250.8***
Total bilirubin µmol/L	*Pre-study*	0.35	0.23	0.08	0.30	0.23	0.30	0.18	0.60
	4	0.98	0.55	0.45	0.25	1.10	0.63	0.65	0.40
	6	0.80	0.53	0.35	0.10	1.48	0.55*	0.45**	0.28**
	12	0.78	1.13	0.80	0.30	1.20	0.53	0.55	0.53

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Absolute and relative liver weights were statistically significantly increased in high-dose males in comparison with controls (by 43% and 56.5% respectively). Relative liver weights were also increased in females and females from the mid dose of 600 ppm.

Table B.6.3-33: Liver weight increases in the 12-month dog study with isoflucypram

Dose levels (ppm)	**Males**				**Females**			
	0	**150**	**600**	**1800**	**0**	**150**	**600**	**1800**
Liver wt (g)	241.8	215.5	266.2	**346.2****	255.6	292.7	271.6	279.0
% difference from controls	-	-	10%	**43%**	-	14.5%	9.2%	9.2%
Relative liver weight	2.53	2.38	**3.12**	**3.96****	2.56	2.69	**3.01**	**3.21**
% difference from controls	-	-	**23.3%**	**56.5%**	-	-	**17.6%**	**25.4%**

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Upon gross necropsy, an enlarged liver was present in 3/4 males and 1/4 females at 1800 ppm and in 1/4 females at 600 ppm; no other macroscopic findings were noted. Histopathological findings were confined to the liver and comprised an increase in the incidence of hepatocellular hypertrophy (centrilobular) in all animals at 1800 ppm and in two males and three females at 600 ppm. Kupffer cell pigmentation (two males and one female), single cell necrosis (one female) and eosinophilic intracytoplasmic inclusion (one male) were also evident at 1800 ppm. The observed microscopic findings corresponded to the increased liver weights and clinical chemistry findings (evidence that the liver is a target organ). The table below summarises the observed macroscopic and microscopic findings.

Table B.6.3-34: Macroscopic and microscopic findings in the 12-month dog study with isoflucypram

Dose levels (ppm)	**Males**				**Females**			
	0	**150**	**600**	**1800**	**0**	**150**	**600**	**1800**
N examined	4	4	4	4	4	3	4	4
Enlarged liver	0	0	0	3	0	0	1	1
Hepatocellular hypertrophy, centrilobular								
Total	0	0	2	4	0	0	3	4
Intracytoplasmic eosinophilic inclusion								

Total	0	0	0	1	0	0	0	0
Kupffer cell pigmentation: focal								
Total	0	0	0	2	0	0	0	1
Single cell necrosis: focal								
Total	0	0	0	0	0	0	0	1

Discussion and conclusion

After 12-months' dietary administration of isoflucypram to Beagle dogs in a standard OECD 452 guideline study, relative liver weights were adversely increased (>15%) in both sexes from 600 ppm; the organ was enlarged in males and females at the top dose (3/4 and 1/4 animals respectively) and also in 1/4 mid-dose females. In addition to hepatocellular hypertrophy from 600 ppm (equivalent to 18.8 and 17.6 mg/kg bw/d) in males and females, other histopathological changes indicative of a more direct toxic effect were noted in the liver at the high-dose of 60/50 mg/kg bw/d in M/F only, including kupffer cell pigmentation, single cell necrosis and cytoplasmic changes. ALP was also increased from the mid-dose. There were also adverse effects on body weight and body weight gain in males from the mid dose and in females at the top dose, and increased salivation at the top dose.

A NOAEL of 150 ppm (equivalent to 4.2 mg/kg bw/d in males and females) is therefore proposed by the RMS as no adverse effects were seen at this dose level. A LOAEL of 600 ppm (18.8/17.6 mg/kg bw/d in males/females) is proposed based on liver weight increases in males and females at the mid- and high-doses and also on enlargement of the liver (with associated hypertrophy) and increased ALP.

([redacted] *2017a)*

B.6.3.3. Other routes

No other routes of exposure were investigated.

B.6.3.4. Discussion and conclusion of short-term toxicity

The short-term toxicity of isoflucypram has been investigated in rats, mice and dogs after 28- and 90-days' oral dietary exposure; a one-year dietary study in dogs is also available. Although not conducted to OECD guidelines, the 28-day studies were broadly similar to these and the 90-day and one-year studies complied with the releveant guidelines. No other routes of exposure have been investigated. The main findings are summarised in table B.6.3.4.1 below. Further information on the repeated dose toxicity of isoflucypram is also available from the 2-generation study (see section B.6.6.1.) and in the chronic studies (see section B.6.5.).

The main target organ of toxicity in all species is the liver. The toxicological significance of the effects on the liver has been assessed by the RMS using a weight-of-evidence approach, with a clear distinction being made between effects that are clearly adverse and those which are potentially adaptive. This has been carried out in line with the TAB (Technical Agreements for Biocides) entry, agreed at the Biocide WG-IV-2018 meeting (WGIV2018_TOX_6-2); a paper which is based on several international reviews of liver effects (JMPR 2006 and 2015) and which describes a weight-of-evidence approach for the evaluation of liver effects in repeated-dose toxicity studies. Hepatocellular hypertrophy is typically related to increased functional capacity of the liver which allows the maintenance of homeostasis in the organism after xenobiotic exposure. A general increase in the size of the liver is observed (owing to cell enlargement and fluid accumulation); this is considered a potentially beneficial, adaptive response. However, there is the potential that the capacity of the homeostatic mechanisms may be exceeded and in these cases the organism would be unable to return to its previous state once exposure has ended (thus constituting an adverse response). Hypertrophy as an adaptive response should not be accompanied by adverse histopathology (necrosis, apoptosis, pigment deposition or hyperplasia), or by substantial changes in clinical chemistry indicative of liver toxicity (decreased albumin or increased activities of ALT, AST, ALP, GGT, bilirubin or cholesterol). In line with the TAB entry, relative liver weight increases up to 15%, that are not accompanied by other signs of liver dysfunction have been considered by the RMS to be an adaptive rather than an adverse response.

Table B.6.3-35: Summary of short-term toxicity studies with isoflucypram

Study	Species	Doses	NOAEL in M/F (mg/kg bw/d)	Main adverse effects
28-day oral (dietary) Non-guideline Non-GLP Isoflucypram Purity 98.6% [redacted] 2017 (SA 11308)	**Rat**, Wistar 5/sex/group	0, 300, 1000 & 3000 ppm Equivalent to: Males: 0, 22.8, 83.3 & 240 mg/kg bw/d Females: 0, 25.6, 86.5 & 285 mg/kg bw/d	**83.3/25.6**	There were no treatment-related deaths or clinical signs of toxicity <u>3000 ppm (240/285 mg/kg bw/d)</u> ↓ bw gain (-32%**) & final bw (-11.4%**) in M ↑ cholesterol (26%*), total protein (6%**) and albumin (8%**) in M ↑ relative liver weight in M (31%**) and F (63%**), ↑ absolute liver weight in F (64%**), ↑ relative thyroid weight in M (41%**) Enlarged liver in M (2/5) & F (5/5), dark liver in M (3/5) & F (2/5), prominent lobulation in liver in F (3/5) Liver: ↑ hepatocellular hypertrophy in 3/5 M (minimal) & 4/5 F (3 minimal, 1 slight), Thyroid: ↑ follicular cell hypertrophy in 2/5 M (slight) & 3/5 F (minimal), Kidney: ↑ basophilic tubules in 3/5 M (minimal), ↑ tubular hyaline droplets in 5/5 M (1minimal, 1 slight, 3 moderate) – not relevant to humans <u>1000 ppm (83.3/86.5 mg/kg bw/d)</u> ↑ relative liver weight in F (30%**), ↑ absolute liver weight in F (27%**) Enlarged liver in M (2/5) & F (1/5), dark liver in F (3/5) Liver: ↑ hepatocellular hypertrophy in 1/5 F (minimal), Thyroid:↑ follicular cell hypertrophy in 1/5 M (minimal) & 1/5 F (minimal), Kidney ↑ basophilic tubules in 3/5 M (2 minimal, 1 slight), ↑ tubular hyaline droplets in 5/5 M (2 minimal, 3 slight) – not relevant to humans <u>300 ppm (22.8/25.6 mg/kg bw/d)</u> Kidney: ↑ tubular hyaline droplets in 3/5 M (2 minimal, 1 slight) – not relevant to humans
28-day oral (dietary) Non-guideline Non-GLP	**Mice**, C57BL/6J 5/sex/group	0, 200, 800 & 2000 ppm Equivalent to:	**32/149**	There were no treatment-related deaths or clinical signs of toxicity <u>2000 ppm (330/374 mg/kg bw/d)</u>

Study	Species	Doses	NOAEL in M/F (mg/kg bw/d)	Main adverse effects
Isoflucypram Purity 98.6% (w/w) [redacted] 2012		Males: 0, 32, 133 & 330 mg/kg bw/d Females: 0, 41, 149 & 374 mg/kg bw/d		↓ bw gain in F (-69%; wk 1; not statistically significant), ↓ food consumption in F (days 1 & 8) ↑ ALAT in M (361%*) & F (110%*), ↑ ASAT (119%*) & ALP (19%) in M ↑ absolute liver weight in M (20%**) & F (30%**), ↑ relative liver weight in M (19%**) & F (25%**) Dark liver in F (3/5) Liver: ↑ hepatocellular hypertrophy in 5/5 M (3 minimal, 2 slight) & 4/5 F (minimal), ↑ hepatocellular necrotic focus in 5/5 M (minimal) & 5/5 F (2 minimal, 3 slight), ↑ hepatocellular single cell necrosis in 5/5 M (slight) & 4/5 F (3 minimal, 1 slight) <u>800 ppm (133/149 mg/kg bw/d)</u> Dark liver in M (1/5) Liver: ↑ hepatocellular necrotic focus in 1/5 M (slight), ↑ hepatocellular single cell necrosis in 2/5 M (minimal) <u>200 ppm (32/41 mg/kg bw/d)</u> No adverse effects
28-day oral dietary US EPA OCSPP 870.SUPP Non-GLP Isoflucypram Purity 98% [redacted] 2014	**Dogs**, Beagle 2/sex/group	0, 300, 1000 & 3000 ppm Equivalent to: Males: 0, 12.7, 37.7 & 76.9 mg/kg bw/d Females: 0, 11.3, 36.5 & 90.2 mg/kg bw/d	A NOAEL was not set from this study owing to the small group sizes used	There were no deaths <u>3000 ppm (76.9/90.2 mg/kg bw/d)</u> ↑ salivation in 1 M (days 15 & 22) ↑ bw losess in 2 M (-0.5 & -0.2 kg) & 1 F (-0.3 kg), ↓ bw gain in 1 F (-91.6%) ↓ final bw in F (-16%) ↓ food consumption in 2 M & 1 F ↑ ALP in 1 M (71%) & 2 F (455 & 191%) ↑ absolute liver weight in M (26%) & F (28%), ↑ relative liver weight in M (34%) & F (52%) Enlarged liver in 1 M Liver: Hepatocellular hypertrophy in 2/2 M (minimal) & 1/2 F (minimal), ↓ severity of hepatocellular glycogen accumulation in M, brown pigments in Kupffer cells in 2/2 F <u>1000 ppm (37.7/36.5 mg/kg bw/d)</u>

Study	Species	Doses	NOAEL in M/F (mg/kg bw/d)	Main adverse effects
				↑ absolute liver weight in F (35%), ↑ relative liver weight in F (39%) <u>300 ppm (12.7/11.3 mg/kg bw/d)</u> ↑ absolute liver weight in F (19% abs; 24.7% rel)
90-day oral (dietary) OECD 408 (1998) GLP Isoflucypram Purity 97.7% [redacted] (2014) (amended 2017)	**Rats**, Wistar 10/sex/group 10/sex/group (control and high-dose) recovery group	0, 100, 300 & 1000 ppm Equivalent to: Males: 0, 6.34, 18.4 & 63.5 mg/kg bw/d Females: 0, 7.92, 21.9 & 80.9 mg/kg bw/d Four week recovery group (control & high-dose)	**18.4/21.9**	There were no treatment-related deaths or clinical signs of toxicity. <u>*Dosing phase*</u> <u>1000 ppm (63.5/80.9 mg/kg bw/d)</u> ↓ total bw gain in M (12%) & F (8%), ↓ final bw in M (8%), ↑ cholesterol in F (21%*) ↑ urinary casts in 5/10 M ↑ absolute liver weights in F (20%*), ↑ relative liver weights in M (11%*) & F (28%**) ↑ relative thyroid weight in M (21%*) Enlarged liver in 2/10 M & 8/10 F Liver: Hepatocellular hypertrophy in 6/10 F (5 minimal, 1 slight) Kidney:Hyaline droplets in kidney tubules in 10/10 M (4 minimal, 5 slight, 1 moderate), basophilic tubules in 6/10 M (1 minimal, 5 slight), granular casts in 2/10 M (minimal) Thyroid: Follicular cell hypertrophy in 5/10 M (minimal) & 5/10 F (minimal), colloid alteration in 5/0 M (minimal) & 2/10 F (minimal) <u>300 ppm (18.4/21.9 mg/kg bw/d)</u> Kidney: ↑ hyaline droplets in 7/10 M (5 minimal, 2 slight) – not relevant to humans <u>100 ppm (6.34/7.92 mg/kg bw/d)</u> No adverse effects <u>*Recovery phase*</u> <u>1000 ppm (63.5/80.9 mg/kg bw/d)</u> ↑ relative liver weight in M (8%) Previous alterations to clinical chemistry, urinalysis & macroscopic findings were reversed during recovery

Study	Species	Doses	NOAEL in M/F (mg/kg bw/d)	Main adverse effects
90-day oral (dietary) OECD 408 (1998) GLP Isoflucypram Purity 97.7% [redacted] (2013)	**Mice**, C57BL/6J 10/sex/group	0, 100, 300 & 1000 ppm Equivalent to: Males: 0, 17, 51 & 168 mg/kg bw/d Females: 0, 19.5, 59.8 & 207 mg/kg bw/d	**51.9/59.8**	There were no treatment-related deaths or clinical signs of toxicity 1000 ppm (168/207 mg/kg bw/d) ↑ absolute liver weights in M (12%*) & F (13%*), ↑ relative liver weights in M (18%**) & F (17%**) Dark liver in 1/10 M & 2/10 F Liver: ↑ hepatocellular vacuolation (centrilobular): 3/10 M (minimal) & 8/10 F (4 minimal, 4 slight), ↓ hepatocellular vacuolation (diffuse): 7/10 M (2 minimal, 6 slight) 300 ppm (51/59.8 mg/kg bw/d) No adverse effects 100 ppm (17/19.5 mg/kg bw/d) No adverse effects
90-day oral (dietary) OECD 409 (1998) GLP Isoflucypram Purity 94.2% [redacted](2015)	**Dogs**, Beagle 4/sex/group	0, 170, 500 & 1500 ppm Equivalent to: Males: 0, 5.5, 15.9 & 50.4 mg/kg bw/d Females: 0, 5.5, 16.2 & 54 mg/kg bw/d	**15.9/16.2**	There were no treatment-related deaths 1500 ppm (50.4/54 mg/kg bw/d) ↑ salivation in 2/4 males ↓ bw gain in M (39%**) & F (39%) ↑ ALP in M & F ↑ relative liver weights in M (42.8%**) ↑ relative liver weights in F (20%) Liver: Centrilobular hepatocellular hypertrophy in 3/4 M (minimal), intracytoplasmic eosinophilic inclusion in 2/4 M 500 ppm (15.9/16.2 mg/kg bw/d) No adverse effects 170 ppm (5.5 mg/kg bw/d) No adverse effects
12-month oral (dietary) OECD 409 (2009) GLP Isopflucypram Purity 94.2%	**Dogs,** Beagle 4/sex/group	0, 150, 600 & 1800 ppm Equivalent to: Males: 0, 4.2, 18.8 & 60.2 mg/kg bw/d Females: 0, 4.2, 17.6 &	**4.2**	There were no treatment-related deaths 1800 ppm (60.2/49.8 mg/kg bw/d) ↑ salivation in 1/4 M Body –weight loss in F (wk 1), ↓ body-weight gain in M (wk 1), ↓ food consumption in F (25%, days 1-7, 10% days 1 to 364)

Study	Species	Doses	NOAEL in M/F (mg/kg bw/d)	Main adverse effects
[redacted] (2017)		49.8 mg/kg bw/d		↑ ALP in M & F ↑ Absolute liver weights in M (43%**), ↑ relative liver weights in M (56.5%**) & F (25.4%) Enlarged liver in 3/4 M & 1/4 F Liver: Hepatocellular hypetrophy in 4/4 M & 4/4 F, intracytoplasmic eosinophilic inclusion in 1/4 M, Kupffer cell pigmentation in 2/4 M & 1/4 F, single cell necrosis in 1/4 F 600 ppm (18.8/17.6 mg/kg bw/d) ↓ body-weight gain and terminal body weight in M, ↑ ALP in M (166%) and F (67%) at month 12 and in M (124%) at month 4, ↑ relative liver weights in M (23.3%) & F (17.6%) Enlarged liver in 1/4 F Lover: Hepatocellular hypertrophy in 2/4 M & 3/4 F 150 ppm (4.2 mg/kg bw/d) No adverse effects

Rat

In rats, the main target organs of toxicity were the liver, thyroid and kidney.

Adverse (> 15%) increased liver weights (with or without hypertrophy) were seen from 86 mg/kg bw/d in the 28-day study and at the top dose of 63 mg/kg bw/d in the 90-day study. In addition, similar effects were seen from 34 mg/kg bw/d in the 2-generation study and at the top dose of 46 mg/kg bw/d in the 2-year carcinogenicity study. These effects were associated with alterations of some clinical-chemistry parameters indicative of liver toxicity (e.g.increased cholesterol) at the top dose of 240 mg/kg bw/d in the 28-day study, at the top dose of 63 mg/kg bw/d in the 90-day study and from 34 mg/kg bw/d in the 2-generation study.

Increased thyroid weights with or without associated histopathology (follicular cell hypertrophy, colloid alterations and pigmentation) were seen at the top dose of 240 mg/kg bw/d in the 28-day study and at the top dose of 63 mg/kg bw/d in the 90-day study. In addition, similar effects were seen from 34 mg/kg bw/d in the 2-generation study and from 19 mg/kg bw/d in the 2-year carcinogenicity study. Mechanistic investigations (see section B.6.8.2) have shown that these changes were associated with increases in TSH and that the most likely MoA (mode of action) underpinning the induction of these effects is activation of CAR/PXR in the liver followed by enzyme induction, including T4-UDPGT, increased clearance of T4, stimulation of the pituitary with increased secretion of TSH leading to stimulation of the thyroid and follicular cell hypertrophy. It is well established that there are large quantitative differences between rats and humans in the regulation of thyroid homeostasis (Colnot & Dekant, 2017). Therefore, these thyroid effects seen in the rat as a secondary consequence of liver enzyme induction are considered to be not relevant to humans.

Kidney histopathology, related to α2u globulin accumulation, was seen in males only from 23 mg/kg bw/d in the 28-day study and at the top dose of 63 mg/kg bw/d in the 90-day study. It was not seen in the 2-generation study up to the top dose of 93 mg/kg bw/d or in the 2-year carcinogenicity study up to the top dose of 19 mg/kg bw/d. The effects in the kidney are male rat-specific and not relevant to humans.

In addition to toxic effects in these organs, decreases in body weight and/or body weight gain were observed at the top dose of 240 mg/kg bw/d in the 28-day study, at the top dose of 63 mg/kg bw/d in the 90-day study and at the top dose of 47 mg/kg bw/d in the 2-year carcinogenicity study.

Mouse

In mice, the main target organs of toxicity were the liver and kidney. There were no effects on the thyroid.

Increased liver weights with associated histopathology (necrosis and/or vacuolation and/or bile-duct hyperplasia) were seen from 133 mg/kg bw/d in the 28-day study, from 168 mg/kg bw/d in the 90-day study and at the top dose of 147 mg/kg bw/d in the 18-month carcinogenicity study. These effects were associated with alterations of some clinical-chemistry parameters indicative of liver toxicity (e.g.increased cholesterol, AST, ALT, etc) at the top dose of 330 mg/kg bw/d in the 28-day study. The effects on the liver in the mouse are more severe than those seen in the rat, but they occur at higher dose levels than the adverse effects observed in the rat.

Increased kidney weights with associated histopathology (hyaline casts, tubule dilatation and basophilia) were seen in male mice and only in the carcinogenicity study only at the top dose of 147 mg/kg bw/d. These kidney effects in the mouse were not associated with α2μ globulin accumulation and are therefore considered relevant to humans.

In addition to toxic effects in these organs, decreases in body weight and/or body weight gain and increased mortality rate (in females) were observed in the carcinogenicity study at the top dose of 147 mg/kg bw/d.

Overall, the mouse appears less sensitive to the toxic effects of isoflucypram compared to the rat.

Dog

In dogs, the main target organ of toxicity is the liver. There were no effects on the thyroid or kidney, providing further evidence supporting the lack of human relevance of the thyroid effects that were seen in the rat (see section B.6.8.2).

Adverse increased liver weights with associated hypertrophy were seen at the top dose of 50 mg/kg bw/d in the 90-day study and from 17 mg/kg bw/d in the 12-month study. More severe histopathological findings (Kupffer cell pigmentation, necrosis and cytoplasmic changes) occurred in the 12-month study at the higher dose of 50 mg/kg bw/d. These effects were associated with increased levels of ALP seen at the top dose of 50 mg/kg bw/d in the 90-day study and from 17 mg/kg bw/d in the 12-month study. The liver hypertrophy seen in the dog after 90-days' exposure is similar to that observed in the rat and it occurred at similar dose levels; however, in the 12-month study, more severe histopthaological findings were noted. In conclusion, adverse effects were seen in the liver after both 90-days and 12-months' exposure in dogs.

In addition to toxic effects in the liver, decreases in body weight and/or body weight gain were at the top dose of 50 mg/kg bw/d in both the 90-day and 12-month study.

Overall, the dog appears to be as sensitive as the rat to the toxic effects of isoflucypram.

When compared with the classification criteria for STOT-RE, the liver was the only target organ at doses below the cut-off values for classification into category 2. However, the RMS concluded that the findings were neither consistent nor severe enough to warrant classification for STOT-RE 2 (see CLH report for further details). The following NOAELs were identified from the repeated-dose toxicity studies:

Table B.6.3-36: Summary of NOAEL values for the repeated-dose toxicity of isoflucypram

Study, guideline, reference	Species, doses tested	NOAEL mg/kg bw/d	LOAEL mg/kg bw/d	Adverse effects at LOAEL
Dietary 28-day No guideline ([redacted]2017)	Rat (Wistar) 0, 300, 100, 3000 ppm Equivalent to 0, 22.8/25.6, 83.3/86.5, 240/285 mg/kg bw/d in M/F	83.3 (M) – 1000 ppm 25.6 (F) – 300 ppm	240 (M) – 3000 ppm 86.5 (F) – 1000 ppm	M (240 mkd):↓ body-weight gain & final body weight; ↑ abse & rel liver weight with associated hypertrophy & ↑ rel thyroid weight with associated follicular cell hypertrophy; effects on clinical-chemistry parameters indicative of liver toxicity; kidney effects (α2uaccumulation) – not relevant to humans F (86.5 mkd): ↑ abs & rel liver weight with associated hypertrophy
Dietary 90-day OECD 408 ([redacted]2014)	Rat (Wistar) 0, 100, 300, 1000 ppm Equivalent to 0, 6.3/7.9, 18.4/21.9, 63.5/80.9 mg/kg bw/d in M/F	18.4 (M) – 300 ppm 21.9 (F) – 300 ppm	63.5 (M) – 1000 ppm 80.9 (F) – 1000 ppm	M&F:↓ body-weight gain; ↑ abs & rel liver weight with associated hypertrophy; ↑ abs & rel thyroid weight with associated follicular cell hypertrophy/colloid alterations; increased cholesterol; kidney histopathology and effects on urinalysis parameters (α2u accumulation) in males only – not relevcant to humans
Dietary 28-day No guideline ([redacted]2012)	Mouse (C57BL/6J) 0, 200, 800, 2000 ppm Equivalent to 0, 32/41, 133/149, 330/374 mg/kg bw/d in M/F	32 (M) – 200 ppm 149 (F) – 800 ppm	133 (M) – 800 ppm 374 (F) – 2000 ppm	M (133 mkd): Hepatocellular necrotic foci, single cell necrosis; ↑ rel liver weight; F (374 mkd): Hepatocellular necrotic foci, single cell necrosis; ↑ rel liver weight; effects on clinical-chemistry indicative of liver toxicity
Dietary 90-day OECD 408 ([redacted]2013)	Mouse (C57BL/6J) 0, 100, 300, 1000 ppm Equivalent to 0, 17/19.5, 51/59.8, 168/207 mg/kg bw/d in M/F	51 (M) – 300 ppm 59.8 (F) – 300 ppm	168 (M) – 1000 ppm 207 (F) – 1000 ppm	M&F: ↑ rel liver weight and associated histopathology (vacuolation)
Dietary 90-day OECD 409 ([redacted]2015)	Dog (Beagle) 0, 170, 500, 1500 ppm	15.9 (M) – 500 ppm 16.2 (F) – 500 ppm	50.4 (M) – 1500 ppm 54 (F) – 1500 ppm	↑ rel liver weight with associated hypertrophy; ↑ ALP; ↓ body-weight gain

Study, guideline, reference	Species, doses tested	NOAEL mg/kg bw/d	LOAEL mg/kg bw/d	Adverse effects at LOAEL
	Equivalent to 0, 5.5/5.5, 15.9/16.2, 50.4/54 mg/kg bw/d in M/F			
Dietary 12-month OECD 452 ([redacted] 2017a)	Dog (Beagle) 0, 150, 600, 1800 ppm Equivalent to 0, 4.2/4.2, 18.8/17.6, 60.2/49.8 mg/kg bw/d in M/F	**4.2 (M & F) – 150 ppm**	18.8 (M) – 600 ppm 17.6 (F) – 600 ppm	↑ rel liver weight with associated hypertrophy, ↑ ALP, enlarged liver

The overall/most sensitive **NOAEL for short-term toxicity** was **4.2 mg/kg bw/d** for males and females (with a respective LOAEL of 18.8/17.6 mg/kg bw/d in males/females) taken from the 12-month dog study. At the LOAEL, there were adverse (>15%) increases in liver weights and in ALP in both sexes.

Overall, therefore, repeated-dose toxicity has been adequately investigated in studies in rats, mice and dogs; the critical target organ was the liver and adverse effects observed in this organ are relevant to humans. Classification for repeated-dose toxicity is not required.

B.6.4. GENOTOXICITY

The genotoxicity of isoflucypram was tested in a *S. typhimurium* reverse mutagenesis assay (Ames test), an *in vitro* V79/HPRT gene mutation assay in Chinese hamster V79 cells, an *in vitro* chromosome aberration study using human lymphocytes and an *in vivo* mouse micronucleus study. The studies were all conducted according to the relevant OECD TGs and were GLP compliant.

B.6.4.1. In vitro studies

Ames test

Study	BCS-CN88460; technical: *Salmonella typhimurium* reverse mutation assay
Reference	Sokolowski, 2014
Date performed	1-16 April 2014
Test facility	Harlan Cytotest Cell Research GmbH, Rossdorf, Germany
Report reference	1614801
Guideline(s)	OECD 471 (1997)
Deviations from the guideline	None
GLP	Yes. Signed QA and GLP certificates provided
Test material	Isoflucypram technical. Batch: 2013-006492, Purity 94.2% w/w
Study acceptable	Yes

Methods

The potential of isoflucypram to induce gene mutations in bacteria was investigated in two experiments using the plate incorporation test (Experiment 1) and the pre-incubation test (Experiment 2) with *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100, and TA 102. The assay was performed, both with and without liver

microsomal (S9) activation. Each concentration, including the controls, was tested in triplicate. The test item was tested at concentrations of 0, 3, 10, 33, 100, 333, 1000, 2500, and 5000 µg/plate.

Results

Precipitation of the test substance was observed but had no influence on scoring of the plates.

The plates incubated with the test item showed normal background growth up to 5000 µg/plate with and without S9 mix in all strains used.

No toxic effects, evident as a reduction in the number of revertants, occurred in the test groups with and without metabolic activation with the exception of strain TA 1537, where minor toxic effects were observed at 1000 and 5000 µg/plate without metabolic activation in Experiment 1 and at 2500 and 5000 µg/plate with metabolic activation in Experiment 2.

No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with isoflucypram at any dose level, either in the presence or absence of metabolic activation. There was no tendency to increased mutation rates with increased concentrations in the range below the generally acknowledged limit of biological relevance. Appropriate reference mutagens were used as positive controls and showed a distinct increase in induced revertant colonies.

Table B.6.4-1: Results from the plate incorporation assay in Salmonella typhimurium strains with isoflucypram

S9 +/-	Test item	Dose / plate	Revertant colony counts (mean ± SD)				
			TA 1535	TA 1537	TA 98	TA 100	TA 102
-	DMSO	-	13 ± 2	9 ± 2	20 ± 7	151 ± 20	445 ± 40
	Untreated	-	13 ± 2	9 ± 3	25 ± 5	170 ± 8	480 ± 14
	Isoflucypram	3 µg	15 ± 2	7 ± 1	24 ± 8	149 ± 17	424 ± 17
		10 µg	15 ± 3	9 ± 1	23 ± 6	142 ± 13	473 ± 51
		33 µg	17 ± 3	9 ± 5	22 ± 5	157 ± 29	466 ± 25
		100 µg	17 ± 2	7 ± 2	24 ± 4	148 ± 18	456 ± 18
		333 µg	12 ± 4[PM]	8 ± 1[PM]	22 ± 5[PM]	163 ± 11[PM]	456 ± 19[PM]
		1000 µg	15 ± 1[PM]	2 ± 1[PM]	20 ± 3[PM]	152 ± 17[PM]	422 ± 12[PM]
		2500 µg	15 ± 1[PM]	5 ± 3[PM]	21 ± 2[PM]	180 ± 11[PM]	460 ± 23[PM]
		5000 µg	14 ± 2[PM]	4 ± 2[PM]	19 ± 2[PM]	175 ± 28[PM]	471 ± 7[PM]
	NaN3	10 µg	2970 ± 236			1729 ± 110	
	4-NOPD	10 µg			272 ± 19		
		50 µg		65 ± 15			
	MMS	2.0 µL					5413 ± 86
+	DMSO	-	13 ± 2	15 ± 2	34 ± 7	167 ± 32	552 ± 74
	Untreated	-	13 ± 2	19 ± 6	48 ± 1	178 ± 14	623 ± 4
	Isoflucypram	3 µg	14 ± 1	19 ± 3	47 ± 4	161 ± 10	662 ± 8
		10 µg	13 ± 1	14 ± 2	35 ± 6	149 ± 22	656 ± 58
		33 µg	13 ± 2	18 ± 9	36 ± 6	150 ± 10	656 ± 87
		100 µg	10 ± 3	25 ± 2	41 ± 4	166 ± 10	621 ± 51
		333 µg	11 ± 2[PM]	13 ± 2[PM]	36 ± 2[PM]	158 ± 4[PM]	511 ± 24[PM]
		1000 µg	10 ± 3[PM]	10 ± 3[PM]	26 ± 3[PM]	156 ± 13[PM]	553 ± 8[PM]
		2500 µg	16 ± 1[PM]	18 ± 3[PM]	38 ± 10[PM]	156 ± 3[PM]	533 ± 13[PM]
		5000 µg	22 ± 1[PM]	14 ± 1[PM]	35 ± 3[PM]	162 ± 6[PM]	504 ± 12[PM]
	2-AA	2.5 µg	549 ± 15	307 ± 27	3304 ± 172	3722 ± 153	
		10.0 µg					3834 ± 153

P = precipitation in medium; M = Manual count

Table B.6.4-2: Results from the pre-incubation assay in Salmonella typhimurium strains with isoflucypram

S9 +/-	Test item	Dose / plate	Revertant colony counts (mean ± SD)				
			TA 1535	TA 1537	TA 98	TA 100	TA 102
-	DMSO	-	17 ± 2	9 ± 3	23 ± 4	131 ± 10	388 ± 22
	Untreated	-	16 ± 3	9 ± 3	26 ± 3	195 ± 4	466 ± 11
	Isoflucypram	3 µg	10 ± 2	10 ± 3	24 ± 4	122 ± 14	447 ± 28
		10 µg	12 ± 4	10 ± 2	24 ± 1	130 ± 3	409 ± 18
		33 µg	19 ± 4	8 ± 4	22 ± 6	134 ± 12	455 ± 22
		100 µg	14 ± 4	11 ± 5	26 ± 5	139 ± 11	427 ± 31
		333 µg	16 ± 4[PM]	15 ± 6[PM]	39 ± 4[PM]	144 ± 8[PM]	415 ± 13[PM]
		1000 µg	14 ± 3[PM]	9 ± 2[PM]	31 ± 2[PM]	144 ± 11[PM]	413 ± 10[PM]
		2500 µg	18 ± 4[PM]	7 ± 3[PM]	28 ± 3[PM]	128 ± 14[PM]	422 ± 14[PM]
		5000 µg	14 ± 3[PM]	9 ± 2[PM]	30 ± 7[PM]	116 ± 15[PM]	384 ± 30[PM]
	NaN3	10 µg	2796 ± 210			1720 ± 208	
	4-NOPD	10 µg			345 ± 38		
		50 µg		67 ± 16			
	MMS	2.0 µL					4324 ± 96
+	DMSO	-	11 ± 3	20 ± 2	38 ± 2	126 ± 7	589 ± 85
	Untreated	-	15 ± 5	20 ± 7	41 ± 13	169 ± 10	563 ± 54
	Isoflucypram	3 µg	14 ± 6	23 ± 3	47 ± 3	122 ± 8	537 ± 59
		10 µg	15 ± 1	20 ± 3	39 ± 8	126 ± 14	492 ± 18
		33 µg	16 ± 3	20 ± 6	47 ± 4	127 ± 20	509 ± 32
		100 µg	14 ± 2	18 ± 2	31 ± 7	129 ± 11	511 ± 47
		333 µg	13 ± 2[PM]	23 ± 3[PM]	35 ± 2[PM]	93 ± 10[PM]	617 ± 20[PM]
		1000 µg	13 ± 2[PM]	16 ± 3[PM]	33 ± 7[PM]	105 ± 16[PM]	502 ± 13[PM]
		2500 µg	15 ± 1[PM]	7 ± 2[PM]	37 ± 4[PM]	105 ± 7[PM]	466 ± 44[PM]
		5000 µg	13 ± 4[PM]	6 ± 2[PM]	26 ± 4[PM]	115 ± 11[PM]	440 ± 39[PM]
	2-AA	2.5 µg	586 ± 1	288 ± 4	2839 ± 546	2895 ± 159	
		10.0 µg					1515 ± 401

P = precipitation in medium; M = Manual count

Conclusion

In conclusion, in a guideline Ames test, isoflucypram did not induce gene mutations in bacteria up to the limit concentration for this test. Therefore, isoflucypram is considered to be non-mutagenic in this *Salmonella typhimurium* reverse mutation assay.

(Sokolowski, 2014)

In vitro chromosome aberration test

Study	BCS-CN88460, technical: Chromosome aberration test in human Lymphocytes *in vitro*
Reference	Bohnenberger, S (2014)
Date performed	26 February – 14 May 2014
Test facility	Harlan Cytotest Cell Research GmbH, Rossdorf, Germany
Report reference	Study number 1614803; Sponsor reference TXLLN075

Guideline(s)	OECD 473
Deviations from the 2016 guideline	Yes – only 100 metaphases per concentration were evaluated vs 300 required by the current guideline. This does not invalidate the study as despite the reduced sensitivity, a positive response was seen.
GLP	Yes. Signed QA and GLP certificates provided
Test material	Isoflucypram technical. Batch: 2013-006492, Purity 94.2% w/w
Study acceptable	Yes

Methods

The potential of isoflucypram to induce structural chromosomal aberrations in human lymphocytes was tested *in vitro* in two independent experiments. The following study design was performed:

Table B.6.4-3: Study design for the in vitro chromosome aberration test in human lymphocytes

	Without S9 mix		**With S9 mix**
	Experiment 1	**Experiment 2**	**Experiments 1 and 2**
Exposure period	4 hours	22 hours	4 hours
Recovery	18 hours	-	18 hours
Preparation interval	22 hours	22 hours	22 hours

In each experimental group, two parallel cultures were analysed and at least 100 metaphases per culture were evaluated for structural chromosomal aberrations, except for the positive control in Experiment 2, in the absence of S9 mix, where only 50 metaphases were evaluated.

The highest applied concentration in the pre-test (4245.0 µg/ml of the test item, approximately 10 mM) was chosen with regard to the molecular weight and the purity (94.2%) of the test item.

Concentration selection for the cytogenetic experiment was performed taking into consideration the cytotoxicity data and the precipitation in the test system of the test item. In experiment 1, concentrations of 0, 7.8, 13.6 and 23.9 µg/mL and 0, 13.6, 23.9 and 41.8 µg/mL were applied, without and with S9 respectivley. In experiment 2, doses of 0, 2, 3.5 and 6.1 µg/mL and 0, 10, 20, 30 and 40 µg/mL were applied, without and with S9 respectivley.

Results

In the absence and presence of S9 mix, clear cytotoxicity was observed at the highest evaluated concentration.

In Experiment 1, in the absence of S9 mix, one single statistically significant increase in chromosomal aberrations (3.3% aberrant cells, excluding gaps), slightly above the range of the laboratory historical solvent control data (0.0-3.0% aberrant cells, excluding gaps), was observed after treatment with 13.6 µg/ml, but not at the top concentration of 23.9 µg/ml. Although no concentration dependency was observed, it is possible that at the top concentration, the presence of cytotoxicity had prevented the expression of genotoxicity. It is also noted that an increase in chromosomal aberrations excluding gaps was seen at the top concentration of 6.1 µg/ml in Experiment 2 (see below) after continuous treatment for 22 hours. No relevant increase was observed in the presence of S9 mix.

In Experiment 2, in the absence of S9 mix, one statistically significant increase in chromosomal aberrations (7.0% aberrant cells, excluding gaps), clearly above the range of laboratory historical solvent control data (0.0-2.5% aberrant cells, excluding gaps), was observed after continuous treatment with the top concentration of 6.1 µg/ml. In the presence of S9 mix, one statistically significant increase in chromosomal aberrations (4.5% aberrant cells, excluding gaps), above the range of the laboratory historical solvent control data (0.0-3.5% aberrant cells, excluding gaps) was observed after treatment with the top concentration of 40.0 µg/ml. At the next lower concentration of 30.0 µg/mL, an increase in chromosomal aberrations without statistical significance (3.8% aberrant cells, excluding gaps), but slightly exceeding the range of the laboratory historical solvent control data (0.0-3.5% aberrant cells, excluding gaps) was also observed.

Overall, the RMS concludes that there was a marginally positive response with S9 (experiment 2) and without S9 (experiment 1 and 2) in this test.

No evidence of an incresae in polyploid metaphases was observed after treatment with the test item as compared to the control cultures.

Appropriate mutagens were used as positive controls. They induced statistically significant increases in the incidence of cells with structural chromosomal aberrations.

Table B.6.4-4: Summary of the results of the chromosomal aberration study with isoflucypram

Exp.	Exposure (h)	S9 +/-	Concentration (µg/ml)	Mitotic index, % control	Incl. gaps	Excl. gaps	Carrying exchanges
1	4	-	Solvent (DMSO)	100.0	0.5	0.5	0.5
			Positive control	76.6	10.5	9.5[S]	3.5
			7.8	83.1	0.5	0.5	0.0
			13.6	88.8	3.8	3.3S	0.0
			23.9	43.2	3.0	2.5	0.0
	4	+	Solvent (DMSO)	100.0	1.5	1.5	1.0
			Positive control	52.2	15.5	15.5[S]	2.5
			13.6	117.8	1.5	1.5	0.0
			23.9	88.9	2.0	1.5	0.5
			41.8	53.3	2.5	2.5	0.0
2	22	-	Solvent control (DMSO)	100.0	1.5	1.5	0.0
			Positive control	49.8	65.0	64.0[S]	12.0
			2.0	84.4	0.5	0.5	0.0
			3.5	92.8	3.5	2.5	0.0
			6.1	45.1	7.0	7.0S	0.0
	4	+	Solvent control (DMSO)	100.0	2.5	1.5	0.0
			Positive control	46.2	25.5	25.0[S]	3.0
			10.0	79.2	3.0	3.0	0.0
			20.0	66.0	1.5	1.5	0.0
			30.0	57.6	4.0	3.8	0.0
			40.0	35.4	5.8	4.5[S]	0.0

S – statistically significant

Conclusion

In conclusion, occasional increases in chromosome damage, mainly manifest as chromatid breaks, were observed with and without metabolic activation, alhough there was no clear evidence of concentration-related responses or consistency across experiments. Overall, in this guideline *in vitro* chromosome aberration test in human lymphocytes, a positive response was observed with and without metabolic activation.

(Bohnenberger, 2014)

Mammalian cell gene mutation in V79(HPRT)

Study	BCS-CN88460, technical: Gene mutation assay in Chinese hamster V79 cells *in vitro* (V79 / HPRT)
Reference	Wollny (2014)
Date performed	25 February – 6 May 2014
Test facility	Harlan Cytotest Cell Research GmbH, Rossdorf, Germany
Report reference	1614802
Guideline(s)	OECD 476 (1997)
Deviations from the 2016 guideline	None
GLP	Yes. Signed QA and GLP certificates provided
Test material	Isoflucypram technical. Batch: 2013-006492, Purity 94.2% w/w

Study acceptable	Yes

Methods

The potential of isoflucypram to induce gene mutations at the HPRT locus in V79 cells of the Chinese hamster was tested *in vitro* in two independent experiments, using identical experimental procedures. In the first experiment, the treatment period was 4 hours with and without metabolic activation (S9). The second experiment was performed with a treatment time of 4 hours with and 24 hours without metabolic activation.

In the pre-experiment, the maximum concentration of 4200 µg/ml was equal to a molar concentration of about 10 mM. The concentration range of the main experiments was limited by precipitation. Without S9, isoflucypram was tested from a concentration of 2.0 to 64.0 µg/ml; with S9, from 4.0 to 128.0 µg/ml. DMSO was used as a solvent. Cytotoxicity was measured by measuring the relative cloning efficiency after the expression period (CE II), also referred to as relative survival (RS).

The guideline (476, 1997) criteria for positive and negative results were as follows:

"A test item is classified as positive (mutagenic) if it induces either a concentration-related increase in the mutant frequency, or a reproducible and positive response at one of the test points. A test item producing neither a concentration-related increase of the mutant frequency, nor a reproducible positive response at any of the test points, is considered non-mutagenic in this system."

Table B.6.4-5: Concentrations of isoflucypram used in the mammalian cell gene mutation assay

	Experiment 1		**Experiment 2**	
	- S9	**+ S9**	**- S9**	**+ S9**
Exposure period, h	4	4	24	4
Concentration of isoflucypram (µg/mL)	2.0	4.0	4.0	4.0
	4.0	8.0	8.0	8.0
	8.0	16.0	16.0	16.0
	16.0	32.0	32.0[P]	32.0
	24.0[P]	48.0	48.0[P]	48.0[P]
	32.0[P]	64.0[P]	64.0[P]	64.0[P]
				96.0[P]
				128.0[P]

Results

The first mutation experiment was conducted with an exposure period of 4 hours, both with and without S9 metabolic activation. In the incubations without metabolic activation, precipitation of the test item was noted at the end of the treatment period at concentrations of 24.0 µg/mL and above. With metabolic activation, precipitation was noted at 64.0 µg/mL and above. No clear cytotoxic effect was observed up to the top concentrations tested.

In the second mutation experiment, with an exposure period of 4 hours in the presence of S9 and 24 hours without S9, precipitation was observed at 32.0 µg/mL and above without S9 and at 48.0 µg/mL and above with S9. No clear cytotoxic effect was observed up to the top concentrations tested.

Overall, no substantial, biologically relevant or reproducible dose-dependent increase in mutation frequency was observed up to the top concentration tested either in the presence of absence of metabolic activation.

Table B.6.4-6: Summary of the results of the HPRT-locus mammalian gene mutation *in vitro* assay with isoflucypram

Treatment	Conc., µg/mL	S9	Culture I			Culture II		
			CE II, %	Mutant colonies / 10^6 cells	Mutation induction factor	CE II, %	Mutant colonies / 10^6 cells	Mutation induction factor
DMSO		-	100.0	3.8	1.0	100.0	3.5	1.0
EMS	150.0		103.4	138.0	36.6	96.0	94.5	26.7
Isoflucypram	4.0		98.8	**15.4**	4.1	99.0	5.0	1.4
	8.0		100.0	9.4	2.5	96.6	3.3	0.9
	16.0		102.0	6.6	1.8	98.5	6.8	1.9
	24.0P		102.9	**27.7**	7.3	97.5	3.8	1.1
	32.0P		100.1	10.6	2.8	95.7	**11.2**	3.2
Solvent HCD[1] (- S9, 4 hr) for mutant colonies			Range: 1.6 – 42.8; mean: 14.9 ± 7.8					
DMSO		+	100.0	6.6	1.0	100.0	6.9	1.0
DMBA	1.1		93.9	177.3	26.8	97.8	221.9	32.0
Isoflucypram	8.0		96.7	7.2	1.1	101.2	5.4	0.8
	16.0		94.6	4.9	0.7	100.9	14.8	2.1
	32.0		97.0	5.5	0.8	99.3	**22.9**	3.3
	48.0		97.8	12.5	1.9	99.7	7.4	1.1
	64.0P		96.8	4.7	0.7	102.6	**20.5**	3.0
Solvent HCD[1] (+ S9, 4 hr) for mutant colonies			Range: 3.4 – 44.2; mean: 14.3 ± 7.1					
DMSO		-	100.0	28.3	1.0	100.0	12.6	1.0
EMS	150.0		99.1	429.1	15.2	100.7	421.3	33.5
Isoflucypram	8.0		101.5	4.8	0.2	104.9	13.2	1.0
	16.0		101.5	9.2	0.3	101.2	12.9	1.0
	32.0P		98.9	6.9	0.2	97.5	10.8	0.9
	48.0P		101.6	12.7	0.4	100.6	6.8	0.5
	64.0P		101.2	30.0	1.1	101.3	16.5	1.3
Solvent HCD[1] (- S9, 24 hr) for mutant colonies			Range: 2.4 – 41.8; mean: 14.1 ± 7.4					
DMSO		+	100.0	22.5	1.0	100.0	15.3	1.0
DMBA	1.1		97.3	115.9	5.2	105.2	147.5	9.6
Isoflucypram	8.0		94.8	5.9	0.3	103.2	15.8	1.0
	16.0		96.4	25.4	1.1	105.0	20.2	1.3
	32.0		65.7	23.0	1.0	105.8	25.2	1.6
	48.0P		99.9	15.8	0.7	102.7	10.8	0.7
	64.0P		94.6	4.4	0.2	105.9	24.7	1.6
	128.0P		100.2	4.2	0.2	102.9	**48.5**	3.2
Solvent HCD[1] (+ S9, 4 hr) for mutant colonies			Range: 3.4 – 44.2; mean: 14.3 ± 7.1					

P = precipitation; CE II = Relative Cloning Efficiency determined after the expression period;
[1] = Lab solvent HCD from 81/82 studies conducted in 2012 and 2013;

In the first experiment, there were substantial increases in the mutation frequency at 4.0 and 24.0 µg/mL without metabolic activation (culture 1); at 32.0 µg/mL (top concentration) without metabolic activation (culture II); and at 32.0 and 64.0 µg/mL (top concentration) with metabolic activation (culture II). However, all of these effects were judged to be biologically irrelevant fluctuations based on the lack of a dose-response (confirmed by negative trend tests). In addition, the mutation frequency remained well within the historical range of solvent controls, and none of these increases was reproduced in the parallel cultures under identical conditions.

In the second experiment, there was only one isolated increase in the mutation frequency at the top concentration of 128 µg/mL in culture II with metabolic activation. This increase exceeded the range of historical solvent

controls. In addition, a trend test was statistically significant in this culture (see below). However, as it was not reproduced in the parallel culture under identical experimental conditions and it occurred far into the precipitating concentration range, it was judged as biologically irrelevant.

A pairwise statistical analysis of the results was not carried out. A linear regression analysis (least squares) was performed to assess a possible concentration-dependent increase in mutation frequencies. A significant concentration dependent trend of the mutation frequency, indicated by a probability value < 0.05, was solely determined in the second culture of the second experiment with metabolic activation. This trend was however judged irrelevant as it was based on the precipitation artefact discussed above.

Appropriate reference mutagens, used as positive controls, induced a distinct increase in mutant colonies and thus showed the sensitivity of the test system and the activity of the metabolic activation system.

Conclusion

In conclusion, in this guideline in vitro test, isoflucypram did not induce gene mutations at the HPRT locus in V79 cells up to concentrations causing precipitation. Isoflucypram is considered to be non-mutagenic in this HPRT assay.

(Wollny, 2014)

B.6.4.2. In vivo studies in somatic cells

Mouse bone marrow micronucleus study

Study	BCS-CN88460, technical - Micronucleus assay in bone marrow cells of the mouse
Reference	[redacted]2014
Date performed	26 February 2014-11 March 2014
Test facility	[redacted]
Report reference	1614802
Guideline(s)	OECD 474
Deviations from the guideline	This study was performed in accordance with the OECD guideline in force at the time it was conducted. It deviates from the current guideline (2016) as follows: 1. Only 2000 polychromatic erythrocytes were examined per animal, rather than 4000 2. Plasma levels of the test substance were not measured nor was any other means of demonstrating exposure of bone marrow required. The applicant has provided an assessment of why these deviations will not impact on the conclusions and validity of this study. The evaluation of the applicant's case by the RMS is provided below.
GLP	Yes. Signed QA and GLP certificates provided
Test material	Isoflucypram technical. Batch: 2013-006492, Purity 94.2% w/w
Study acceptable	Yes

Methods

This study was performed to investigate the potential of isoflucypram to induce micronuclei in polychromatic erythrocytes in the bone marrow of the mouse.

The test item was dissolved in DMSO/PEG 400 (3/7), which was also used as the vehicle control. Both test item and vehicle control were administered twice orally with an interval of 24 hours. The volume administered on each occasion was 10 ml/kg bw/day (total volume of applications was 20 ml/kg bw). At 48 hours after the first

administration of the test item (24 hours after the last treatment) the bone marrow cells were collected for micronuclei analysis.

Seven males per test group were evaluated for the occurrence of micronuclei with 2000 polychromatic erythrocytes (PCEs) scored for micronuclei per animal.

To determine whether the treatment had had a cytotoxic effect, the ratio between polychromatic and normochromatic erythrocytes was determined in the same sample and reported as the number of PCEs per 2000 erythrocytes.

Based on pre-experiments, 2000 mg isoflucypram/kg bw administered twice orally (2 x 2000 mg/kg bw) was suitable as the highest treatment dose. Thus, the dose levels of 500, 1000, and 2000 mg/kg bw twice orally were investigated in the mutagenicity experiment.

Results

Clinical signs of toxicity (reduction of spontaneous activity, ruffled fur, apathy, etc.) were seen in a dose-related manner at all dose levels. This may indicate systemic exposure to isoflucypram and/or its metabolites.

Table B.6.4-7: Clinical signs of toxicity observed in male mice after gavage administration of a first and second dose of isoflucypram in the micronucleus study

Dose	Observation		Time after treatment, in hours			
		Application	0-1	2-4	5-6	24
500 mg/kg bw	Reduction of spontaneous activity	1	3	0	0	0
		2	0	1	0	0
	Ruffled fur	1	4	4	4	3
		2	4	4	4	3
1000 mg/kg bw	Reduction of spontaneous activity	1	5	5	0	0
		2	0	0	0	0
	Abdominal position	1	1	0	0	0
		2	0	0	0	0
	Eyelid closure	1	2	0	0	0
		2	0	0	0	0
	Ruffled fur	1	5	5	6	4
		2	5	5	7	4
	Apathy	1	1	0	0	0
		2	0	0	0	0
2000 mg/kg bw	Reduction of spontaneous activity	1	6	4	0	0
		2	0	0	0	0
	Abdominal position	1	1	0	0	0
		2	0	0	0	0
	Eyelid closure	1	2	0	0	0
		2	0	0	0	0
	Ruffled fur	1	7	7	7	5
		2	7	7	7	5
	Apathy	1	1	0	0	0
		2	0	0	0	0
	Excitement	1	0	0	0	0
		2	1	0	0	0

After treatment with the test item, the number of PCEs was not substantially decreased as compared to the mean value of PCEs of the vehicle control, thus indicating that isoflucypram did not exert any measureble cytotoxic effects in the bone marrow. In comparison to the corresponding vehicle control, there was no biologically relevant or statistically significant increase in the frequency of the detected micronuclei at any preparation interval and any dose level. A dose of 40 mg/kg bw cyclophosphamide administered once orally was used as a positive control and showed a substantial increase of induced micronucleus frequency.

Table B.6.4-8: Incidence of micronucleated PCEs in male mice administered isoflucypram at up to 2000 mg/kg bw

	Isoflucypram, dietary concentration in mg/kg bw/d				Cyclophosphamide, 40 mg/kg bw
	0	500	1000	2000	
PCEs with micronuclei, %	0.093	0.093	0.086	0.129	1.850
Range	1-3	0-4	1-2	0-4	22-68
PCE/2000 erythrocytes	1242	1202	1174	1202	1116
Significance		n.t.	n.t.	-	+
P value		-	-	0.1906	0.003

- not significant
+ significant
n.t.: not tested as the micronucleus frequency was not above the vehicle control value

Evaluation of the impact of deficiencies on the validity of the study

1. Under the 1997 OECD test guideline 474, only 2000 polychromatic erythrocytes were required to be examined per animal, in contrast to the 4000 currently required. Given that there was no relevant increase in the incidence of micronucleated PCEs at any dose up to 2000 mg/kg bw, it is unlikely that the results would have differed if 4000 PCEs were examined per animal as is required by the 2016 OECD test guideline 474.

2. This study was conducted in 2014 under the 1997 version of the relevant OECD test guideline 474, which did not require assessment of plasma levels of the test substance, nor was any other means of demonstrating exposure of bone marrow required. The study was therefore in compliance with the OECD test guideline in force at the time the study was conducted. In its 2017 Scientific Opinion (doi: 10.2903/j.efsa.2017.5113), EFSA specified that several lines of evidence could be considered in determining whether or not the bone marrow had been exposed to the test item. These lines of evidence, and the data available for each of them with isoflucypram, are considered below.

   - *Isoflucypam and /or its metabolites detected in plasma in the mouse chronic study:*
     In the mouse chronic study, plasma samples from the 12-month and 18-month time-points showed relatively high concentrations of two metabolites of isoflucypram as well as low concentrations of the parent compound itself ([redacted] 2017; see section B.6.5). As the bone marrow is a highly-perfused tissue, detection of the test item and/or its metabolites in blood or plasma indicates that they will have reached the bone marrow.

   - *Clinical signs of toxicity observed in the mouse bone marrow micronucleus test:*
     Clinical signs of toxicity including abdominal position, ruffled fur, reduction in spontaneous activity, apathy, and/or excitement were observed in all treated groups in a dose-related manner. These clinical signs may indicate systemic toxicity of isoflucypram after oral gavage administration in the mouse and in turn, potential systemic availability of isoflucypram and/or its metabolites in the actual mouse micronucleus test.

   - *Systemic toxicity observed in repeated dose toxicity studies in mice*
     In the mouse 28-day study (see section B.6.3) and chronic study (see section B.6.5), there were effects on body weight (from a dose of 330 mg/kg bw/d in the 28-day study and at the top dose of 150 mg/kg bw/d in the chronic study). These systemic effects may indicate systemic availability of isoflucypram and/or its metabolites in the mouse.

   - *Isoflucypram (and/or metabolites) detected in the bone marrow in a toxicokinetic study in the rat:*
     In a whole body autoradiography study ([redacted] 2017c) described in the ADME section above (B.6.1.1), radioactivity was observed in the bone marrow of rats following oral gavage administration of radiolabelled isoflucypram. This demonstrates bone marrow exposure in the rat.

   - *Isoflucypram (and/or metabolites) detected in blood/urine in a toxicokinetic study in the rat:*
     Both isoflucypram and a number of metabolites were detected in blood and urine after oral gavage administration of radiolabelled isoflucypram to male and female rats in two ADME studies ([redacted] 2017b and 2017c) described in section B.6.1.1 above. This demonstrates systemic availability of isoflucypram and/or its metabolites in the rat.

The above summary shows that several of the lines of evidence proposed in the EFSA 2017 Scientific Opinion "*Clarification of some aspects related to genotoxicity assessment*" for demonstrating that a test item has reached the target tissue in an *in vivo* study can be used in the case of isoflucypram. Thus, it can be accepted that isoflucypram reached the bone marrow in the present *in vivo* mouse micronucleus study, and this study is acceptable under the aforementioned EFSA Scientific Opinion.

Conclusion

In summary, in a guideline micronucleus study, isoflucypram did not induce any increase in the incidence of micronucleated PCEs in the bone marrow of male mice administered the test substance up to the limit dose of 2000 mg/kg bw, at which clinical signs of toxicity occurred. Although there was no direct evidence of bone marrow exposure in the study, in the chronic mouse study, isoflucypram and two major metabolites were detected in plasma, demonstrating systemic availability in the mouse. As the bone marrow is a highly-perfused tissue, it must be inferred that exposure of the bone marrow in the mouse occurred. In addition, the occurrence of clinical signs of toxicity in the micronuclues study itself may indicate that the test substance (and/or its metabolites) was systemically available in this test. Furthermore, there were systemic effects (decreases in body weight) in mouse repeated dose toxicity studies, indicating again potential systemic availability of isoflucypram and/or its metabolites in the mouse. It is also noted that kinetic studies in the rat showed that the substance and/or its metabolites were systemically available and reached the bone marrow in the rat. Thus, it can be concluded that isoflucypram is not clastogenic or aneugenic in a valid *in vivo* mouse micronucleus assay.

([redacted] 2014)

B.6.4.3. In vivo studies in germ cells

As isoflucypram was negative *in vivo* in a mutagenicity test in somatic cells, no *in vivo* mutagenicity studies in germ cells were conducted or are required.

B.6.4.4. Summary of genotoxicity

The genotoxicity of isoflucypram was tested in a S. typhimurium reverse mutagenesis assay (Ames test), an *in vitro* V79/HPRT gene mutation assay in Chinese hamster V79 cells, an *in vitro* chromosome aberration study using human lymphocytes and an *in vivo* mouse micronucleus study. The studies were all conducted according to the relevant OECD TGs and were GLP compliant.

Isoflucypram did not induce gene mutations in bacteria or mammalian cells *in vitro* but was clastogenic *in vitro* with and without metabolic activation. However, when tested *in vivo* in a valid mouse bone marrow micronucleus study up to the limit dose of 2000 mg/kg bw at which clinical signs of toxicity occurred, the clastogenic activity seen in vitro was not expressed *in vivo*. Although there was no direct evidence of bone marrow exposure in the study, in the chronic mouse study, isoflucypram and two major metabolites were detected in plasma, demonstrating systemic availability in the mouse. As the bone marrow is a highly-perfused tissue, it must be inferred that exposure of the bone marrow in the mouse occurred. In addition, the occurrence of clinical signs of toxicity in the micronuclues study itself may indicate that the test substance (and/or its metabolites) was systemically available in this test. Furthermore, there were systemic effects (decreases in body weight) in mouse repeated dose toxicity studies, indicating again potential systemic availability of isoflucypram and/or its metabolites in the mouse. It is also noted that kinetic studies in the rat showed that the substance and/or its metabolites were systemically available and reached the bone marrow in the rat. Thus, it can be concluded that isoflucypram is not clastogenic or aneugenic in a valid *in vivo* mouse micronucleus assay.

Overall, it can be concluded that isoflucypram is not genotoxic *in vivo* and the data requirements of Regulation 283/2013 have been met. Classification of isoflucypram for mutagenicity is not warranted (see also aligned CLH report). A summary of all the available genotoxicity studies is shown in the table below.

Table B.6.4-9: Summary of genotoxicity studies with isoflucypram

Study	Concentrations of Substance tested	Result	Reference
***In vitro* assays**			

Study	Concentrations of Substance tested	Result	Reference
Ames test	0, 3, 10, 33, 100, 333, 1000, 2500, 5000 µg/plate	negative	Sokolowski, 2014
Chromosomal aberrations study in human lymphocytes *in vitro*	Concentrations evaluated: Without S9, exp.1: 0, 7.8, 13., 23.9 µg/mL; exp.2: 0, 2.0, 3.5, 6.1 µg/mL With S9, exp.1: 0, 13.6, 23.9, 41.8 µg/mL; exp.2: 0, 10.0, 20.0, 30.0, 40.0 µg/mL	Clastogenic with and without S9	Bohnenberger, 2014
V79 / HPRT mammalian mutagenicity study	Without S9, exp.1 (4 hours): 0, 4.0, 8.0, 16.0, 24.0, 32.0 µg/mL; exp. 2 (24 hours): 0, 8.0, 16.0, 32.0, 48.0, 64.0 µg/mL. With S9, exp.1 (4 hours): 0, 8.0, 16.0, 32.0, 48.0, 64.0 µg/mL; exp.2 (4 hours): 0, 8.0, 16.0, 32.0, 48.0, 64.0, and 128.0 µg/mL	Negative	Wollny, 2014
***In vivo* assay**			
Mouse micronucleus assay *in vivo*	0, 500, 1000, and 2000 mg/kg bw	Negative	[redacted]2014

B.6.5. LONG-TERM TOXICITY AND CARCINOGENESIS

The long-term toxicity and carcinogenic potential of isoflucypram have been investigated in rats and mice for 2-years and 18-months respectively, in standard OECD guideline dietary studies.

B.6.5.1. Long-term toxicity and carcinogenicity in rats

The long-term toxicity and carcinogenicity of isoflucypram have been investigated in rats via the oral (dietary) route in a standard guideline two-year study.

Study	BCS-CN88460 Chronic toxicity and carcinogenicity study in the Wistar rat by dietary administration
Reference	[redacted](2018)
Test facility	[redacted]
Report reference	SA 13266
Guideline(s)	OECD 453 (2009)
Deviations from the guideline	None
GLP	Yes
Test material	Isoflucypram, Batch : 2013-006492, purity 94.2%
Study acceptable	Yes

Methods

Isoflucypram was administered via the diet to 70/sex/dose male and female Wistar rats (the same strain was used in the previous repeated-dose toxicity studies), for a period of 12-months (chronic phase) or 24-months (carcinogenicity phase). The following dose-levels were selected on the basis of the previous 90-day toxicity study in rats (see section 3.6.2.1). A validated method of analysis for isoflucypram in the diet at the different

concentrations tested is available (see document CA_B5 for further details). It is noted that the dose formulations were not verified on every occasion; however, as the findings and associated dose-responses from this study are consistent with those of the previous 90-day study in the rat, in which the dose formulations were verified on every occasion, it is concluded that the method of analysis of this 2-year study is acceptable.

Table B.6.5-1: Mean test substance intake in the rat chronic/carcinogenicity study with isoflucypram

	Males			**Females**		
Dose level (ppm)	**30**	**150**	**450**	**30**	**150**	**800**
Chronic phase (12-months) mg/kg bw/d	1.416	7.17	21.3	1.968	9.68	52.1
Carcinogenicity phase (2-years) mg/kg bw/d	1.237	6.27	18.6	1.746	8.54	46.6

The top-dose selected for this study is markedly lower than the highest dose tested in the previous 90-day toxicity study, particularly in male rats (450 ppm; equivalent to 21.3 and 18.6 mg/kg bw/d at 12- and 24-months respectively). The applicant has provided the following justification for the criteria used in the selection of this dose which has been reproduced verbatim below:

The doses used in the rat 2-year carcinogenicity study were selected on the basis of the 90-day study ([redacted] 2017), in which BCS-CN88460 was administered to male and female Wistar rats at dietary concentrations of 0, 100, 300, and 1000 ppm, equivalent to approximately 0, 6.34, 18.4, and 63.5 mg/kg bw/day in males, and 0, 7.92, 21.9, and 80.9 mg/kg bw/day in females.

- *At 1000 ppm*
  - *Mean absolute body weight gain was reduced by 12% in males and 8% in females relative to controls, while mean absolute body weight was reduced by 7% in males and unchanged relative to controls in females.*
  - *Mean total bilirubin was decreased in both males and females, and total cholesterol was slightly increased in females only. Although decreased total bilirubin is not an adverse effect, it can be considered an indication of liver enzyme activity.*
  - *Relative liver weight was increased in males, and both absolute and relative liver weights were increased in females. Periportal to panlobular hepatocellular hypertrophy was observed in females, but not in males.*
  - *Relative thyroid weight was increased in males, but no effect on thyroid weight was observed in females. However, an increased incidence of follicular cell hypertrophy and colloid alteration was observed in both male and female rats.*
  - *Cellular casts in the urine were observed in 5 of 10 males.*
  - *Relative kidney weight was increased in males, although this observation is considered related to the slightly lower mean terminal body weight. There was an increase in the incidence and severity of hyaline droplets in proximal tubules and of bilateral basophilic tubules, as well as granular casts in some animals.*

- *At 300 ppm:*
  - *No effect on body weight or body weight gain in either sex.*
  - *Mean total bilirubin was decreased in females only.*
  - *Cellular casts were observed in the urine of one male, although as this was within the historical data it was considered to be non-adverse. In the kidney, there was a slightly higher incidence and severity of hyaline droplets in the proximal tubules, however this observation was considered not to be adverse as it was also seen at the same severity in some control males.*
  - *No effects on organ weights in either males or females.*

- *At 100 ppm:*
  - *The only treatment-related change noted was a decrease in total bilirubin concentrations in females.*

During dose selection for the chronic study, it was considered that extended administration of high dietary concentrations of BCS-CN88460 would exacerbate the kidney findings in males, increasing the mortality related to chronic progressive nephropathy and risking the validity of the study. Based on the urinalysis and

histopathologic findings at 300 and 1000 ppm after 90 days, it was considered that a dose of 450 ppm would induce significant but not excessive toxicity in males without inducing excessive mortality, and this was selected as the high dose in males.

The increased absolute and relative liver weights in females at 1000 ppm in the 90-day study, along with the increase in hepatocellular hypertrophy, were considered to indicate a significant effect on the liver, a known target organ for BCS-CN88460 and other members of its class. The increase in thyroid follicular hypertrophy and colloid alteration at this dose are again known effects of BCS-CN88460 and other similar compounds. These effects on known target organs were used to establish a top dose in females at 800 ppm, where it was expected that organ weight, histopathological findings, and possibly tumor incidence would be increased.

Other doses of 150 and 30 ppm were selected with a view to ensuring roughly equivalent dose spacing, as well as to having an expected No Observable (Adverse) Effect Level at 30 ppm and a dose with some toxicity at the mid dose of 150 ppm.

The RMS accepts the approach taken by the applicant in determining the doses for the combined chronic and carcinogenicity study in rats. The guidance for dose-selection as outlined in the OECD guidance document 116 (on the conduct and design of chronic toxicity and carcinogenicity studies), advises that consideration is given to "the potential that an effect may limit the sensitivity of a chronic carcinogenicity study to detect tumours due to an increase in mortality or severe toxicity that may compromise the health of the animals" and "the potential of a toxic effect to progress in severity when the duration of exposure is increased." The chronic progressive nephropathy observed in male rats in the 90-day study fulfilled both' these criteria; therefore, to ensure that the study was not invalidated by high mortality rates, it was necessary to select a top-dose in males that was markedly lower than that of the previous 90-day study. The rationale for the selection of the top-dose in females (based on effects on target organs at 1000 ppm) is also acceptable.

Results

From each group, 10 males and 10 females were allocated to the chronic phase and necropsied after 52-weeks' exposure; the remaining 60 animals in each group made up the carcinogenicity phase of the study (two-years). The stability and homogeneity of the test-substance in the diet was confirmed in a separate study using a validated method of analysis. . In addition, the plasma concentrations of isoflucypram were determined at months 3-4, 12 and 24 of the study; whilst the plasma concentrations of two of the key metabolites were determined at months 12 and 24 (see toxicokinetics section for results).

For the first 12-months (chronic phase) mortality was low overall and was similar across control and treated groups. Isoflucypram treatment had no effect on mortality during the carcinogenicity phase and the mortality rates amongst treated males during this phase were actually lower than the corresponding controls (see table B.6.5.2). Consequently, all males were sacrificed at week 102, as the mortality rate in the control group approached the 75% threshold for early study termination as outlined in OECD TG 453 (i.e. less than 25% survival in the low-dose or control groups). Females were allowed to go to the full-term of the study and the slightly early termination of male rats did not affect the validity of the study in the opinion of the RMS. No explanation has been provided in the study report for the high mortality rate observed in all groups at the end of the 2 years. Although the mortality rate was high after 2 years in all groups, including controls (56.7% - 71.7% in males; 51.7% - 66.7% in females), mortality was below 25% (survival > 75%) after 18 months and below 50% (survival > 50%) after 21 months in all groups. The mortality rate raised up to levels of 50-70% only in the last 3 months of the study. Therefore, it is the view of the RMS that the power of the study to detect a carcinogenic effect was not compromised. This is more or less in agreement with the recommendation of the OECD guidance document 116 which states that survival should ideally be no less than 50% in all groups by 24 months in rats.

Table B.6.5-2: Mortality rates in the rat carcinogenicity study over 2 years

Dose level (ppm)	**Males**				**Females**			
	0	**30**	**150**	**450**	**0**	**30**	**150**	**800**
Chronic Phase								
Initial N	70	70	70	70	70	70	70	70
Killed for humane reasons	2	-	4	2	2	2	-	5
Found dead	1	-	2	1	-	-	1	-
Died during anesthesia	-	-	-	-	1	-	-	-
Accidental trauma	1	-	-	-	-	-	-	-
Total deaths	4	-	6	3	3	2	1	5

Dose level (ppm)	Males				Females			
	0	**30**	**150**	**450**	**0**	**30**	**150**	**800**
% mortality after 12 months	**5.7%**	**-**	**8.6%**	**4.3%**	**4.3%**	**2.9%**	**1.4%**	**7.1%**
Carcinogenicity phase								
Initial N	60	60	60	60	60	60	60	60
Killed for humane reasons after 2 years	13	16	22	17	29	24	28	25
Found dead after 2 years	30	22	15	17	4	12	12	6
Died during anesthesia after 2 years	-	-	-	-	1	-	-	-
Accidental trauma after 2 years	-	-	-	-	-	-	-	-
Total deaths after 2 years	43	38	37	34	33	36	40	31
% mortality after 2 years	**71.7%**	**63.3%**	**61.7%**	**56.7%**	**55.9%**	**60.3%**	**66.7%**	**51.7%**
Total deaths after 18 months	14	11	12	11	10	7	4	12
% mortality after 18 months	**23%**	**18%**	**20%**	**18%**	**17%**	**12%**	**7%**	**20%**
Total deaths after 21 months	27	23	25	22	16	14	18	19
% mortality after 21 months	**45%**	**38%**	**42%**	**37%**	**27%**	**23%**	**30%**	**32%**

Treatment-related clinical signs of toxicity were confined to females and encompassed an increased incidence of hair loss at the high-dose in both the chronic and the carcinogenicity phases. Overall, there were no effects of treatment on mortality. However, a clinical sign of toxicity (hair loss) was seen in females at the top dose.

Isoflucypram did not have a marked effect on body-weight development (see table B.6.5.3). There were no statistically significant effects on body weight or body-weight gain in male rats at any dose, nor in female rats at the mid- and low-doses. The body weights of high-dose females, however, were generally lower than controls, reaching a maximum of 5% lower on day 344; this led to lower body-weight gain values for these females (from study day 50), which were only occasionally statistically significant and exhibited no clear dose-response (maximum of 10% lower than controls on study days 1-344). This effect was likely to be a consequence of lower food-consumption, which was evident at 800 ppm in females and followed a similar pattern to the slight body-weight reductions in these animals (i.e. always below 5% and only occasionally statistically significant); nonetheless, the effect on body-weight gain in females rats at the high-dose is considered by the RMS to be related to treatment with isoflucypram and adverse. Food consumption was not affected in any other group and there was no effect on water intake. Overall, there were adverse effects on body weight gain in females at the top dose.

B.6.5-3: Body weight development in the two-year rat study with isoflucypram

Dose level (ppm)		Males				Females			
	Day	**0**	**30**	**150**	**450**	**0**	**30**	**150**	**800**
Body weight (g)	1	214.1	213.6	213.6	214.5	165.0	165.6	165.9	165.7
	29	391.7	391.4	389.5	387.7	241.5	243.5	245.4	239.6
	91	533.7	537.2	536.2	530.6	295.1	295.8	301.1	291.1
	316	670.4	673.7	667.7	664.7	352.7	354.1	358.6	**337.1* (4%)**
	344	681.6	681.2	679.9	671.7	359.2	357.4	365.6	**339.5**(5%)**
	540	700.8	722.8	714.2	708.6	417.5	416.1	439.1	394.3
	708	665.4	695.8	670.6	699.0	446.1	470.2	456.2	435.4
	729					454.5	468.4	464.9	442.7
Body-weight gain (g)	1-29	177.6	177.8	175.9	173.2	76.5	77.9	79.5	74.0
	1-91	319.8	323.6	322.4	316.1	130.0	130.2	135.2	125.4 (4%)
	1-344	467.6	467.7	466.9	457.1	194.0	191.6	199.6	**174.0** (10%)**
	1-540	488.6	508.6	501.2	495.8	253.6	251.7	273.3	231.0 (9%)
	1-708	456.3	479.8	460.5	488.8				
	1-729					292.0	304.3	303.3	280.5 (4%)

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

No treatment-related changes to haematology or urinalysis parameters were noted and the only clinical chemistry finding was a reduction in bilirubin (see table B.6.5.4). Such an effect was observed in the majority of the preceding short-term toxicity studies and although treatment-related was not considered adverse. The effect was likely to be a consequence of increased removal of bilirubin, owing to the induction of xenobiotic clearing enzymes. This provides some indication that increased activity of liver enzymes is occurring in this carcinogenicity study, in particular UDGPT-bilirubin, the induction of which was demonstrated in the 28-day rat study.

Table B.6.5-4: Mean total bilirubin in male and female rats administered isoflucypram for up to two-years

Month	Dose levels (ppm)	Males				Females			
		0	30	150	450	0	30	150	800
3/4		1.35	1.21	1.34	0.64**	1.75	1.66	1.64	0.66**
7		1.35	1.21	1.34	1.02*	2.04	1.94	1.87	1.00***
12		1.75	1.48	2.07	1.28*	2.87	2.87	2.80	1.29**
18		1.99	1.65	1.94	1.27	2.80	2.33	1.86	1.27***
24		2.03	1.63	1.97	1.42	2.05	1.95	1.80	1.01**

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

At 12 months (chronic phase), absolute and relative liver weights were increased by 18% and 11% respectively in females at 800 ppm (52.1 mg/kg bw/d). At the end of the carcinogenicity phase, only relative liver weights were increased in comparison with controls; in females at 800 ppm (equivalent to 46.6 mg/kg bw/d during this phase) relative liver weights were 12% greater than the control value. There were no treatment-related effects on any other organs, and the liver weights of males (at all doses) and of females (at the lower doses) were not affected by treatment with isoflucypram (see table B.6.5.5 below). Overall, adverse (>15%) increased absolute liver weight was seen at the top dose in females.

Table B.6.5-5: Liver weight changes in the two-year rat study with isoflucypram

Dose levels (ppm)	Males				Females			
	0	30	150	450	0	30	150	800
12m, number examined	8	10	9	10	10	8	10	10
Terminal body wt, g	684.0	674.7	659.4	654.0	340.3	366.8	342.1	362.8
Liver wt, g	13.1	13.4	12.9	13.4	7.2	7.8	7.6	**8.6***
% change	-	2%	-2%	3%	-	8%	5%	**18%**
Liver wt / body wt, %	1.9	2.0	2.0	2.1	2.1	2.1	2.2	**2.3***
% change	-	4%	2%	8%	-	0%	5%	**11%**
24m, number examined	17	22	23	26	26	24	20	29
Terminal body wt, g	626.9	661.8	639.1	663.1	430.3	440.8	430.1	417.5
Liver wt, g	12.3	13.2	12.5	13.2	9.5	9.8	9.6	10.3
% change	-	8%	2%	8%	-	2%	0%	8%
Liver wt / body wt, %	2.0	2.0	2.0	2.0	2.2	2.2	2.2	**2.5****
% change	-	2%	0%	3%	-	0%	1%	**12**

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

There were no treatment-related macroscopic findings after either 12- or 24-months' exposure. All microscopic observations during the chronic phase of the study (12 months) were common findings amongst rats of this strain and age and were therefore not related to treatment with isoflucypram. Microscopic findings for the carcinogenicity phase (2 years) are summarised in table B.6.5.6. Treatment-related microscopic findings resulting from slight but prolonged stimulation of the thyroid were seen as follows: colloid alteration was observed in males and females of the high-dose group (18.6 and 46.6 mg/kg bw/d respectively) and diffuse pigmentation of the follicular cells was seen in males at 450 ppm (18.6 mg/kg bw/d). These findings in the thyroid are considerd to be adverse by the RMS. No histopathological signs of kidney nephropathy were seen at any dose and there were no microscopic findings in the liver. Overall, thyroid histopathological findings were seen at the top dose in both sexes.

Table B.6.5-6: Microscopic findings (thyroid) at 24-months

Dose levels (ppm)	Males				Females			
	0	30	150	450	0	30	150	800
N examined	59	59	59	59	60	60	60	59

Dose levels (ppm)	Males				Females			
	0	**30**	**150**	**450**	**0**	**30**	**150**	**800**
Colloid alteration								
Minimal	21	16	21	21	10	6	18	23
Slight	6	8	8	16	1	0	1	5
Moderate	0	0	0	1	0	0	0	0
Total	27	24	29	38	11	6	19	**28*** **
Pigmentation, follicular cells, diffuse								
Minimal	2	3	6	11	2	0	0	5
Total	2	3	6	**11***	2	0	0	5

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

After two years' administration of isoflucypram to rats, there were no treatment-related neoplastic findings. There were no increases (relative to controls) in the tumour incidence in any of the target organs identified in the repeated-dose studies nor in any of the potentially endocrine sensitive tissues. Any incidences seen did not show a clear dose-response, were small in magnitude or were also seen in controls. Selected tumour incidences (non-treatment related; identified target organs and endocrine sensitive tissues) are summarised in table B.6.5-7 below.

Table B.6.5-7: Selected neoplastic findings (non-treatment related) in the chronic/carcinogenicity rat study with isoflucypram

Dose-levels (ppm)		Males				Females			
		0	**30**	**150**	**450**	**0**	**30**	**150**	**800**
Epididymis	*N examined*	*70*	*62*	*62*	*70*				
Total tumour incidence[1]		1	2	1	2				
Kidneys	*N examined*	*70*	*70*	*70*	*70*	*70*	*70*	*70*	*70*
Total tumour incidence[2]		0	1	1	1	3	2	0	1
Liver	*N examined*	*70*	*70*	*70*	*70*	*70*	*70*	*70*	*70*
Total tumour incidence[3]		3	4	2	7	2	1	6	3
Mammary glands	*N examined*	*69*	*59*	*59*	*69*	*70*	*62*	*60*	*70*
Total tumour incidence[4]		2	2	2	1	40	36	48	22
Ovaries	*N examined*	-	-	-	-	*70*	*66*	*64*	*70*
Total tumour incidence[5]		-	-	-	-	9	13	16	12
Thyroid gland	*N examined*	*69*	*69*	*69*	*69*	*70*	*70*	*70*	*69*
Total tumour incidence[6]		16	9	11	11	15	14	11	9
Uterus	*N examined*	-	-	-	-	*70*	*67*	*62*	*70*
Total tumour incidence[7]		-	-	-	-	21	34	26	3

[1] Includes M-fibrosarcoma, malignant M-mesothelioma, N-histiocytic sarcoma and N-lymphoma
[2] Includes B-adenoma, B-lipoma, B-transitional cell papilloma, M-carcinoma, N-histiocytic sarcoma and N-lymphoma
[3] Includes B-hepatocellular adenoma, M-hepatocellular carcinoma, N-endometrial adenocarconima, N-histiocytic sarcoma, N-lymphoma, N-mesothelioma, malignant and N-sarcoma
[4] Includes B-adenoma, B-fibroadenoma, M-adenocarcinoma, M-adenocarcinoma, M-carcinosarcoma, M-sarcoma, N-histiocytic sarcoma and N-lymphoma
[5] Includes B-granulosa cell tumor,B-leiomyoma, B-luteoma, B-mixed sex cord stroma tumor, B-tubulostromal adenoma, M-cystadenocarcinoma, M-granulosa cell tumor, M-tubulostromal adenocarcinoma, M-yolk sac carcinoma and N-lymphoma
[6] Includes B-C cell adenoma, B-follicular cell adenoma, M-C cell carcinoma and N-lymphoma
[7] Includes B-endometrial adenoma, B-endomeetrial stromal polyp, B-granular cell tumor, M-endometrial adenocarcinoma, M-hemangiosarcoma, M-Schwannoma, N-histiocytic sarcoma, N-lymphoma and N-yolk sac carcinoma

Discussion and conclusion

Isoflucypram was administered via the diet of male and female Wistar rats for two-years, in a standard guideline study. No increase in the incidence, severity or onset of tumours was observed up to and including the highest-dose tested (450 ppm/18.6 mg/kg bw/d in males and 800 ppm/46.6 mg/kg bw/d in females) at which some limited generalised toxicity (hair loss, decreased body weight gain and increased absolute liver weight in females and minimal thyroid histopathological findings in both sexes) occurred. Therefore, the NOAEL for carcinogenicity in the rat is 18.6/46.6 mg/kg bw/d in M/F.

Some toxicity was observed in females at 800 ppm (equivalent to 46.6 mg/kg bw/d) which indicated that the top-dose was sufficiently high in these animals; body weight was reduced by a maximum of 5% on day 344, and body-weight gain in females was a maximum of 10% lower than controls (for days 1-344). Consistent with the findings of the previous repeated-dose studies, the liver was identified as a target organ in female rats; absolute liver weight was increased by 18% in the top dose females, but no associated histopathology was found. In addition, minimal

thyroid histopathological changes (colloid alteration and diffuse pigmentation of the follicular cells) were seen at the top dose in both sexes.

In conclusion, there were adverse effects on body weight gain and absolute liver weight in females at the top dose of 450/800 ppm (18.6/46.6 mg/kg bw/d in M/F) and minimal thyroid histopathological changes (colloid alteration and diffuse pigmentation of the follicular cells) in both sexes at the top dose. Therefore, the NOAEL for systemic chronic toxicity in the rat is 150 ppm (6.27/8.54 mg/kg bw/d in M/F).

B.6.5.2. Long-term toxicity and carcinogenicity in mice

The chronic toxicity and carcinogenic potential of isoflucypram has been investigated in a guideline study in mice over 18-months' dietary exposure.

Study	BCS-CN88460 – Carcinogenicity study in the c57bl/6j mouse by dietary administration
Reference	[redacted] 2017
Test facility	[redacted]
Report reference	SA 13273
Guideline(s)	OECD 451 (2009)
Deviations from the guideline	None
GLP	Yes
Test material	Isoflucypram, batch : 2013-006492, purity 94.2% (w/w)
Study acceptable	Yes

Methods

Isoflucypram was incorporated in the diet of C57BL6J mice (60/sex/group) for 52-weeks or 18-months. Doses were selected based on the previous 90-day dietary toxicity study in mice, in which minimal effects (decreased bilirubin, increased liver weights and hepatocellular vacuolation) were observed at the high-dose of 1000 ppm. Therefore, owing to the small magnitude of effects observed at 1000 ppm, doses of 0, 50, 250 and 1250 ppm were selected for the carcinogenicity study in mice (equivalent to 0, 6.1, 30.2 and 154 mg/kg bw/d in males and 0, 8.1, 39.6 and 197 in females at 52 weeks and 0, 5.9, 29 and 147 mg/kg bw/d in males and 0, 7.8, 38.1 and 190 mg/kg bw/d in females at 18 months). After 52-weeks' exposure, 10 males and 10 females from each dose group were sacrificed and evaluated for the chronic phase of the study, whilst the remaining 50/sex/dose animals were allocated to the carcinogenicity phase, during which dosing was continued until the conclusion of the 18-month study. The stability and homogeneity of the test item in the diet were demonstrated in a separate study using a validated method of analysis. A validated method of analysis for isoflucypram in the diet at the different concentrations tested is available (see document CA_B5 for further details). It is noted that the dose formulations were not verified on every occasion; however, as the findings and associated dose-responses from this study are consistent with those of the previous 90-day study in the mouse, in which the dose formulations were verified on every occasion, it is concluded that the method of analysis of this carcinogenicity study is acceptable.

In addition, the plasma levels of isoflucypram were determined at 3-, 12- and 18-months, whilst plasma concentrations of two of the major metabolites of isoflucypram were determined at 12- and 18-months (see toxicokinetics section for results).

Results

There were no treatment-related clinical signs in either sex throughout the duration of the study; all observed signs were common for the age and strain of mice, did not show a clear dose-response and occurred in a small number of animals. Overall survival rates were acceptable. In females at 1250 ppm (190 mg/kg bw/d) there was a slight, but non-statistically significant increase in mortality rates (30% compared with 12% in controls) during the carcinogenicity phase (see table B.6.5.8). Mortality rates were also slightly higher than controls at 50 and 250 ppm (7.8 and 38.1 mg/kg bw/d respectively), but clear dose-response was not seen at these doses. Male mortality was not affected by treatment with isoflucypram at any dose-level. Overall, the RMS considers the higher mortality rate seen in females at the top dose treatment-related and adverse.

Table B.6.5-8: Mortality in male and female mice administered isoflucypram for 18-months

Dose-levels (ppm)	Males				Females			
	0	**50**	**250**	**1250**	**0**	**50**	**250**	**1250**
Initial N, day 0	50	50	50	50	50	50	50	50

Dose-levels (ppm)	Males				Females			
	0	**50**	**250**	**1250**	**0**	**50**	**250**	**1250**
Killed for humane reasons	9	6	6	8	6	10	9	14
Found dead	0	0	0	1	0	2	1	1
Total dead	9	6	6	9	6	12	10	15
% mortality	18%	12%	12%	18%	12%	24%	20%	30%

Mean body weights were up to 6% lower than controls at 1250 ppm in both sexes (147 and 190 mg/kg bw/d in males and females respectively) and statistical significance was achieved at most measurements from day 71 (see table B.6.5.9). This resulted in overall mean body-weight gains that were 11/13% lower than controls in males/females. Only minor differences in body-weight development were noted at the lower doses which showed no clear dose-response, and although occasionally statistically significant, were likely to reflect natural individual variation. There was no effect on food consumption. The slight but not excessive toxicity demonstrated by the body-weight gain reductions in male and female treated mice, indicates that adequate dose-levels were reached to allow the assessment of the carcinogenic potential of isoflucypram in mice. Overall, there were adverse effects on body weight and body weight gain at the top dose in both sexes.

Table B.6.5-9: Body-weight development of mice in the 18-month carcinogenicity study with isoflucypram

		Males				Females			
	Day	**0**	**50**	**250**	**1250**	**0**	**50**	**250**	**1250**
Body weight (g)	1	20.63	20.57	20.58	20.51	16.77	16.87	16.88	16.83
	8	21.98	21.98	21.70	21.63	18.00	18.03	17.73	17.41**
	92	27.90	27.47	27.74	27.05**	22.36	21.96	21.71**	21.23**
	176	30.61	30.63	30.61	29.20**	24.07	23.87	23.83	22.86**
	365	32.80	32.52	33.25	31.33†	26.98	27.02	26.86	25.43**
	540	33.11	33.19	32.95	31.72**	27.92	27.87	27.74	26.52*
Body-weight gain (g)	1-8	1.35	1.41	1.12*	1.12*	1.23	1.16	0.85**	0.57**
	1-92	7.28	6.90	7.14	6.54**	5.58	5.09**	4.83**	4.40**
	92-176	2.71	3.15*	2.83	2.15**	1.71	1.91	2.12*	1.64
	176-365	2.12	1.72	2.53	2.06	2.89	3.14	3.00	2.47
	365-540	0.30	0.54	-0.15	0.35	0.91	0.97	0.84	0.93
	1-540	12.51	12.49	12.35	**11.14****	11.15	10.88	10.89	**9.66****
% difference					**11%**				**13%**

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; † $p \leq 0.001$.

No clinical-chemistry parameters were measured in this study and the haematological parameters measured showed no treatment-related differences in relation to the control values.

At 12 months, liver weights in males at the high-dose of 1250 ppm (equivalent to 154 mg/kg bw/d) were increased in comparison with controls by 14% (absolute) and 20% (relative); in females at this dose (197 mg/kg bw/d) and phase, only relative liver weights were statistically significantly greater than controls (by 14%). No effect was observed on liver weights at the mid- and low-dose levels at 12-months. The RMS considers that the absolute and relative liver weight increases observed at the 12-month interim sacrifice are related to treatment with isoflucypram, but only in males at 1250 ppm (154 mg/kg bw/d) are they considered adverse (see section B.6.3.4 for the rationale behind this assessment). No other organs were affected at the interim kill.

B.6.5-10: Selected organ weight changes (liver) in mice after 12-months' exposure with isoflucypram

Dose-levels (ppm)	Males				Females			
	0	**50**	**250**	**1250**	**0**	**50**	**250**	**1250**
Number examined	9	9	9	10	10	9	10	9
Terminal body weight (g)	29.1	29.1	30.6	27.6	23.5	23.8	22.9	22.4
Liver weight (g)	1.169	1.204	1.237	1.338**	1.084	1.128	1.107	1.179
% change	-	3%	6%	14%	-	3%	5%	14%
Relative liver weight (%)	4.030	4.134	4.059	**4.845 *****	4.611	4.741	4.842	5.270**
% change	-	3%	1%	**20%**	-	3%	5%	14%

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; ***$p \leq 0.001$.

After 18-months' exposure (carcinogenicity phase), absolute and relative liver weights in males were increased at 1250 ppm (equivalent to 147 mg/kg bw/d) by 10% and 17% respectively, whilst in females at this dose (equivalent to 190 mg/kg bw/d) relative liver weights were 11% greater than controls. No effect was noted on liver weights at the lower-doses at 18-months. The RMS considers that the relative liver weight increases observed in males and females at 1250 ppm after 18-months are related to treatment with isoflucypram and are adverse in nature, owing to the large magnitude of the increase and/or accompanying histopathological signs (necrosis).

At 18 months, absolute and relative kidney weights were found to be increased in males at 1250 ppm (147 mg/kg bw/d) in comparison with controls by 9% and 16% respectively; accompanying histopathological findings were evident, hence the RMS considered the increased relative kidney weights in males at this dose to be treatment-related and adverse.

The only other changes in organ weights were seen in the adrenal glands at 18 months. At 1250 ppm in males this organ was increased by 22% (absolute) and 30% (relative); a 22% increase in absolute adrenal weight was also seen at 50 ppm but without a corresponding effect on relative weight. In high-dose females, only the relative adrenal weights were affected, being 13% greater than controls. There were no histopathological correlates noted in the adrenal gland and an effect on the adrenal gland was not seen in any other studies, so therefore the RMS concludes that the weight increases seen in this organ are an incidental finding and not related to treatment with isoflucypram.

Overall, adverse increased liver weights were seen at the top dose in both sexes and increased kidney weights were seen at the top dose in males.

B.6.5-11: Seleceted organ weight changes after 18 months in mice treated with isoflucypram

Dose levels (ppm)	**Males**				**Females**			
	0	**50**	**250**	**1250**	**0**	**50**	**250**	**1250**
Number examined	41	44	44	41	44	38	40	35
Terminal body weight (g)	29.12	29.13	28.93	27.42 ***	25.01	24.78	24.69	23.41**
% change				-6%				-6%
Liver weight (g)	1.200	1.266	1.200	1.322 ***	1.273	1.253	1.329	1.322
% change	-	6%	0%	10%***	-	-2%	4%	4%
Relative liver weight (%)	4.129	4.375	4.157	**4.822 *****	5.089	5.065	5.393	5.651 ***
% change	-	6%	1%	**17%**	-	0%	6%	11%
Adrenal weight (g)	0.00410	0.00416	0.00446	**0.00502 *****	0.00787	0.00792	0.00782	0.00836
% change	-	22%	9%	**22%**	-	-	-	-
Relative adrenal weight (%)	0.01416	0.01439	0.01556	**0.01840 ****	0.03162	0.03227	0.03188	**0.03589 ****
% change	-	-	10%	**30%**	-	-	-	**13%**
Kidney weight (g)	0.4915	0.4996	0.5175*	**0.5336 ****	0.4039	0.3937	0.3875	0.3804
% change	-	2%	5%*	**9%**	-	-3%	-4%	-6%
Relative kidney weight (%)	1.6857	1.7082	1.7916 **	**1.9471 *****	1.6166	1.5981	1.5711	1.6269
% change	-	2%	6%*	**16%**	-	-1%	-3%	1%

*: p≤0.05; **: p≤0.01; ***: p≤0.001

There were no macroscopic findings at 12 months, but an enlarged liver was observed in the high-dose groups at 18 months.

Non-neoplastic microscopic findings were observed in the liver of both sexes at the 12 month sacrifice at the top-dose. In males this presented as minimal multinucleated hepatocytes and in females as minimal diffuse bile-duct hyperplasia (see table B.6.5.12).

Table B.6.5-12: Non-neoplastic microscopic findings (liver) in mice at 12-months

	Males				Females			
Dose-levels (ppm)	**0**	**50**	**250**	**1250**	**0**	**50**	**250**	**1250**
N examined	10	10	10	10	10	10	10	10
Multinucleated hepatocytes								
Minimal	1	0	0	8	0	0	1	0
Total	1	0	0	**8****	0	0	1	0
Bile duct hyperplasia: diffuse								
Minimal	1	0	0	0	0	0	1	5
Slight	0	0	0	0	0	0	1	0
Total	1	0	0	0	0	0	2	**5****

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

A similar pattern of non-neoplastic findings was observed in high-dose males and females at 18-months; however, the incidence and severity of the effects in both sexes had progressed at 18-months relative to the 12-month sacrifice. In males the multinucleated hepatocytes were accompanied by a greater incidence (relative to controls) of single cell necrosis and in females the observed bile-duct hyperplasia was accompanied by a greater incidence of hepatocellular necrotic foci as well as a lower incidence and severity of diffuse hepatocellular vacuolation (relative to controls).

B.6.5-13: Non-neoplastic microscopic findings (liver) in mice at 18-months

	Males				Females			
Dose-levels (ppm)	**0**	**50**	**250**	**1250**	**0**	**50**	**250**	**1250**
N examined	**50**	**50**	**50**	**50**	**50**	**50**	**50**	**50**
Multinucleated hepatocytes								
Minimal	3	1	4	28	0	0	0	0
Slight	0	0	0	14	0	0	0	0
Moderate	0	0	0	1	0	0	0	0
Total	3	1	4	**43*****	0	0	0	0
Bile duct hyperplasia: diffuse								
Minimal	5	7	5	2	8	8	9	19
Slight	0	1	0	3	0	1	0	6
Total	5	8	5	5	8	9	9	**25*****
Single cell necrosis: focal								
Minimal	4	0	2	8	5	2	3	2
Slight	0	0	0	2	0	0	1	0
Total	4	0	2	**10***	5	2	4	2
Hepatocellular necrotic focus(i)								
Minimal	11	9	7	14	12	9	13	22
Slight	0	0	0	1	0	0	0	2
Total	11	9	7	15	12	9	13	**24***
Hepatocellular vacuolation: diffuse								
Minimal	29	29	31	27	13	15	15	18
Slight	8	8	5	8	25	21	21	12
Moderate	0	0	0	1	3	1	2	1
Total	37	37	36	36	41	37	38	31

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Non-neoplastic microscopic findings were noted in the kidneys of male mice at 1250 ppm (147 mg/kg bw/d) at the 18-month sacrifice, and comprised an increase in the incidence of hyaline casts, tubule dilation in the medulla and a slight increase in the severity of focal tubule basophilia compared with controls (see table B.6.5.14).

Table B.6.5-14: Non-neoplastic findings (kidneys) in mice at 18-months

	Males				Females			
Dose-levels (ppm)	**0**	**50**	**250**	**1250**	**0**	**50**	**250**	**1250**
N examined	50	50	50	50	50	50	50	50
Hyaline casts: focal								
Minimal	38	31	34	42	41	34	45	42

	Males				Females			
Dose-levels (ppm)	**0**	**50**	**250**	**1250**	**0**	**50**	**250**	**1250**
N examined	50	50	50	50	50	50	50	50
Slight	3	5	4	3	5	7	4	5
Moderate	0	0	0	1	1	0	0	0
Total	41	36	38	46**	47	41	49	47
Tubule dilation: medulla: focal								
Minimal	0	0	1	4	8	3	3	3
Total	0	0	1	4*	8	3	3	3
Tubule basophilia: focal								
Minimal	47	43	39	30	43	37	44	42
Slight	3	7	7	14	3	6	2	1
Moderate	0	0	1	2	0	0	0	0
Marked	0	0	0	1	0	0	0	0
Total	50	50	47	47	46	43	46	43

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

A slight increase in the severity and/or incidence of amyloid deposition was noted in several organs of both sexes at 1250 ppm; the significance of this finding is unclear, but amyloid deposition is known to be a common age-related finding in certain genetically pre-disposed mouse strains, and so it is likely that this is an incidental finding and not a consequence of treatment with isoflucypram.

Overall, liver histopathological findings (minimal multinucleated hepatocytes, single cell necrosis, hepatocellular necrotic foci and minimal diffuse bile-duct hyperplasia) were seen at the top dose in both sexes and kidney histopathological changes (hyaline casts, tubule dilation in the medulla and a slight increase in the severity of focal tubule basophilia) were seen in males at the top dose.

After mice were exposed to isoflucypram for 18-months, there were no treatment-related increases in the incidence, severity or onset of tumours. There was no indication of an increase in tumours in the previously identified target organs in mice, nor in any endocrine sensitive tissues. A selection of tumour incidences (non-treatment related) for target organs and endocrine sensitive tissues are summarised in table B.6.5.15 below.

Table B.6.5-15: Selected neoplastic findings (non-treatment related) in the 18-month mouse study with isoflucypram.

		Males				Females			
		0	**50**	**250**	**1250**	**0**	**50**	**250**	**1250**
Kidneys	*N examined*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*
Total tumour incidence[1]		0	0	0	0	1	0	1	2
Liver	*N examined*	*60*	*60*	*60*	*60*	*60*	*60*	*60*	*60*
Total tumour incidence[2]		5	7	4	3	5	4	7	10
Ovaries	*N examined*					*50*	*50*	*50*	*50*
Total tumour incidence[3]						1	1	3	2
Testis	*N examined*	*50*	*50*	*50*	*50*				
B-Leydig cell adenoma		0	0	1	0				
Thyroid gland	*N examined*	*60*	*50*	*50*	*60*	*60*	*50*	*50*	*60*
B-follicular cell adenoma		0	0	0	0	0	2	0	1
Uterus	*N examined*					*50*	*50*	*50*	*50*
B-endometrial stromal polyp						0	1	0	0
B-glandular polyp						1	0	0	1
B-hemangioma						1	0	0	0
M-leiomyosarcoma						0	0	1	0
N-histiocytic sarcoma						0	0	0	2
N-lymphoma						1	0	1	0
Total tumour incidence[4]						3	1	2	3
Hematopoietic system	*N examined*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*51*
M-histiocytic sarcoma		1	1	3	0	1	2	1	3
M-lymphoma		8	5	4	4	5	7	13	11

[1]includes N-histiocytic sarcoma and N-lymphoma

[2]includes B-hepatocellular adenoma, M-hemangiosarcoma, M-hepatocellular carcinoma, N-histiocytic sarcoma, N-lymphoma

[3]includes B-granulosa cell tumor, B-tubulostromal adenoma, M-granulosa cell tumor, N-histiocytic sarcoma and N-lymphoma
[4]includes B-endometrial stromal polyp, B-glandular polyp, B-hemangioma, M-leiomyosarcoma, N-histiocytic sarcoma and N-lymphoma

Discussion and conclusion

Isoflucypram was administered to male and female mice over 18-months' dietary exposure. There were no treatment-related increases in the incidence, severity or onset of tumours in any tissue up to the top dose of 1250 ppm (147/190 mg/kg bw/d in M/F) at which systemic toxicity (higher mortality rate in females, adverse effects on body weight and body weight gain in both sexes, increased liver weight with associated histopathology in both sexes and increased kidney weight with associated histopathology in males) occurred. Overall, a NOAEL of 1250 ppm (147/190 mg/kg bw/d in M/F) can be identified for carcinogenicity in mice from this study.

The highest dose tested of 1250 ppm (147 and 190 mg/kg bw/d in males and females respectively) was sufficient to induce some (but not excessive) toxicity in both sexes. Increased mortality was noted in females. Reductions in body-weight gain of 11% and 13% in males and females respectively were noted at this dose. Also observed at the high-dose were treatment-related increases in the weights of several organs with histopathological correlates; relative liver weights were increased by 20% in males (accompanied by multinucleated hepatocytes and single cell necrosis). Relative liver weights in females were increased by 14% which is below the 15% cut off value for adversity set by the RMS (see repeated dose-section); however, given the profile of effects seen in males and that histopathological correlates indicative of liver toxicity were evident (hepatocellular necrotic focus and bile-duct hyperplasia) the increase in relative liver weight in females is also considered by the RMS to be treatment-related and adverse.

Relative kidney weights were increased in males at 1250 ppm (by 16%), accompanied by hyaline casts, tubule dilation and an increase in the severity of tubule basophilia; the RMS considers the increased relative kidney weights and histopathological correlates in males at 1250 ppm (147 mg/kg bw/d) to be both treatment-related and adverse.

The proposed NOAEL for systemic chronic toxicity is therefore 250 ppm (equivalent to 29 and 38.1 mg/kg bw/d in males and females respectively) as there were no adverse findings at this dose. At the LOAEL of 1250 (equivalent to 147 and 190 mg/kg bw/d in males and females respectively) there were higher mortality rates in females, reductions in overall body-weight gain of >10%, increased liver and kidney weights and histopathological correlates.

B.6.5.3. Summary and conclusion of chronic toxicity and carcinogenicity

The long-term toxicity and carcinogenic potential of isoflucypram has been investigated in rats and mice in dietary studies of 2-years and 18-months respectively; both were conducted in compliance with OECD test guidelines (453 for rats and 451 in mice). There were no neoplastic findings in either species; the main non-neoplastic findings are summarised in table B.6.5.16 below.

Table B.6.5-16: Summary of long-term and carcinogenicity studies with rats and mice

Study	**Species**	**Doses**	**NOAEL in M/F (mg/kg bw/d)**	**Main adverse effects**
24-month toxicity and carcinogenicity study (dietary) OECD 453 (2009) GLP	Rats, Wistar Rj:WI(IOPS HAN) Chronic phase: 10/sex/dose (12 months) Carcinogenicity phase: 60/sex/dose (24 months)	Males: 0, 30, 150 & 450 ppm Equivalent to: Chronic phase: 0, 1.416, 7.17 & 21.3 mg/kg bw/d Carcinogenicity phase: 0, 1.237, 6.27 & 18.6 mg/kg bw/d	**18.6/8.54**	**Chronic phase – 12 months** 450/800 ppm (21.3/52.1 mg/kg bw/d) ↑ incidence of hair-loss in F ↓ BW-gain in females (10%** days 1-344) ↑ liver weight in F (18%*absolute & 11%* relative) 150 ppm (7.17/9.68 mg/kg bw/d) No adverse effects 30 ppm (1.416/1.968 mg/kg bw/d)

<table>
<tr><th>Study</th><th>Species</th><th>Doses</th><th>NOAEL in M/F
(mg/kg bw/d)</th><th>Main adverse effects</th></tr>
<tr><td></td><td></td><td>Females: 0, 30, 150 & 800
Equivalent to:
Chronic phase: 0, 1.968, 9.68 & 52.1 mg/kg bw/d
Carcinogenicity phase: 0, 1.746, 8.54 & 46.6 mg/kg bw/d</td><td></td><td>No adverse effects
<u>Carcinogenicity phase – 24 months</u>
Non-neoplastic findings
<u>450/800 ppm (18.6/46.6 mg/kg bw/d)</u>
↑ relative liver weight in F (12%**)
Thyroid: ↑ incidence of colloid alteration in F (28*** compared with 11 in controls), ↑ incidence of follicular cell pigmentation in M (11* compared with 2 in controls)
<u>150 ppm (6.27/8.54 mg/kg bw/d)</u>
No adverse effects
<u>30 ppm (1.237/1.746 mg/kg bw/d)</u>
No adverse effects
Neoplastic findings
Isoflucypram is not carcinogenic in rats</td></tr>
<tr><td>18-months carcinogenicity study (dietary)
OECD 451 (2009)
GLP</td><td>Mice, C57BL/6J
Chronic phase: 10/sex/dose (12 months)
Carcinogenicity phase: 50/sex/dose (18 months)</td><td>0, 50, 250 & 1250 ppm
Equivalent to:
Males:
0, 6.1, 30.2 & 154 mg/kg bw/d (chronic phase) & 0, 5.9, 29 & 147 mg/kg bw/d (carcinogenicity phase)
Females:
0, 8.1, 39.6 & 197 mg/kg bw/d (chronic phase) & 0, 7.8, 38.1 & 190 mg/kg bw/d (carcinogenicity phase)</td><td>29/38.1</td><td><u>Chronic phase – 12 months</u>
<u>1250 ppm (154/197mg/kg bw/d)</u>
↓ BW in M (4%***) & F (6%**)
↑ absolute liver weight M & F (14%**)
↑ relative liver weight in M (20%***) & F (14%)
Liver: ↑ incidence of multinucleated hepatocytes in M (8** compared with 1 in control), ↑ incidence of bile-duct hyperplasia in F (5** compared with 0 in controls)
<u>250 ppm (30.2/39.6 mg/kg bw/d)</u>
No adverse effects
<u>50 ppm (6.1/8.1 mg/kg bw)</u>
No adverse effects
<u>Carcinogenicity phase – 18 months</u>
Mortality at 18 months:
↑ mortality rate in top dose females
<table>
<tr><th rowspan="2">Dose level (ppm)</th><th colspan="2">Mortaility %</th></tr>
<tr><th>Males</th><th>Females</th></tr>
<tr><td>0</td><td>18%</td><td>12%</td></tr>
<tr><td>50</td><td>12%</td><td>24%</td></tr>
</table></td></tr>
</table>

Study	Species	Doses	NOAEL in M/F (mg/kg bw/d)	Main adverse effects
				250 | 12% | 20% 1250 | 18% | **30%** *Non-neoplastic findings* <u>1250 ppm (147/190 mg/kg bw/d)</u> ↓ BW in M (4%**) & F (6%*) ↓ BWG in M (11%**) & F (13%**) ↑ absolute liver weight in M (10%***) ↑ relative liver weight in M (17%***) & F (11%***) ↑ absolute (9%***) & relative (16%***) kidney weight in M Liver: ↑ incidence of microscopic findings in M (multinucleated hepatocytes & single cell necrosis) and F (bile-duct hyperplasia & necrotic focus) Kidney: ↑ incidence of microscopic findings in M (hyaline casts, tubule dilation & tubule basophilia) <u>250 ppm (29/38.1 mg/kg bw/d)</u> No adverse effects <u>50 ppm (5.9/7.8 mg/kg bw)</u> No adverse effects *Neoplastic findings* Isoflucypram is not carcinogenic in mice

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; BW = body weight ; BWG = body-weight gain

In rats, no increase in the incidence, severity or onset of tumours was observed up to and including the highest-dose tested (450 ppm/18.6 mg/kg bw/d in males and 800 ppm/46.6 mg/kg bw/d in females) at which some limited generalised toxicity (hair loss, decreased body weight gain and increased absolute liver weight in females and minimal thyroid histopathological findings- colloid alteration and diffuse pigmentation of the follicular cells- in both sexes) occurred. No other systemic effects occurred at lower doses. Therefore, isoflucypram was not carcinogenic at the highest dose tested of 450/800 ppm (18.6/46.6 mg/kg bw/d in M/F). The NOAEL for systemic chronic toxicity is 150 ppm (6.27/8.54 mg/kg bw/d in M/F).

In mice, there were no treatment-related increases in the incidence, severity or onset of tumours in any tissue up to the top dose of 1250 ppm (147/190 mg/kg bw/d in M/F) at which systemic toxicity occurred (higher mortality rate in females, adverse effects on body weight and body weight gain in both sexes, increased liver weight with associated histopathology, including necrosis and bile-duct hyperplasia in both sexes and increased kidney weight with associated histopathology, including hyaline casts, tubule dilation and tubule basophilia in males) occurred. No other systemic effects occurred at lower doses. Overall, isoflucypram was not carcinogenic in mice at the highest dose tested of 1250 ppm (147/190 mg/kg bw/d in M/F). The NOAEL for systemic chronic toxicity is 250 ppm (29/38.1 mg/kg bw/d in M/F).

Overall, therefore, isoflucypram is not carcinogenic in rats or mice. No classification for carcinogenicity is warranted (see aligned CLH report).

The following NOAELs have been identified for the chronic toxicity and carcinogenicity of isoflucypram.

Table B.6.5-17: Summary of NOAEL values for the chronic toxicity/carcinogenicity of isoflucypram

Study, guideline, reference	**Species, Doses tested**	**NOAEL mg/kg bw/d**	**LOAEL mg/kg bw/d**	**Adverse effects at the LOAEL**
Dietary 24-month OECD 453 ([redacted] 2018)	Rat (Wistar) 0, 30, 150, 450/800 ppm Equivalent to 0, 1.2/1.7, 6.27/8.54, 18.6/46.6 mg/kg bw/d in M/F	*Carcinogenicity* 18.6 (M) – 450 ppm 46.6 (F) – 800 ppm	*Carcinogenicity* >18.6 (M) – 450 ppm >46.6 (F) – 800 ppm	*Carcinogenicity* No tumours observed up to the top-dose
		Chronic toxicity **6.27 (M) – 150 ppm** 8.54 (F) – 150 ppm	*Chronic toxicity* 18.6 (M) – 450 ppm 46.6 (F) – 800 ppm	*Chronic toxicity* Hair loss (F) ↓ body weight gain (F) ↑ abs liver weight (F) Minimal thyroid histopath changes (M & F)
Dietary 18-month OECD 451 ([redacted] 2017)	Mouse (C57BL/6J) 0, 50, 250, 1250 ppm Equivalent to 0, 5.9/7.8, 29/38.1, 147/190 mg/kg bw/d in M/F	*Carcinogenicity* 147 (M) – 1250 ppm 190 (F) – 1250 ppm	*Carcinogenicity* >147 (M) – 1250 ppm >190 (F) – 1250 ppm	*Carcinogenicity* No tumours observed up to the top dose
		Chronic toxicity 29 (M) – 250 ppm 38.1 (F) – 250 ppm	*Chronic toxicity* 147 (M) – 250 ppm 190 (F) – 250 ppm	*Chronic toxicity* ↑ mortality rate (F) ↓ body weight and body weight gain (M & F) ↑ liver weight and histopathological correlates (M & F) ↑ kidney weight and histopathological correlates (M)

The overall/most sensitive **NOAEL for chronic toxicity** is **6.7 mg/kg bw/d** identified in the rat 2-year study. Effects at the LOAEL of 18.6/46.6 mg/kg bw/d (M/F) included hair loss, decreased body weight gain and increased absolute liver weight in females and minimal thyroid histopathological changes in both sexes.

B.6.6. REPRODUCTIVE TOXICITY

The reproductive toxicity of isoflucypram has been investigated in a standard guideline dietary 2-generation study in rats and in guideline gavage pre-natal developmental toxicity studies, one in rats and one in rabbits.

B.6.6.1. Generational studies

The reproductive toxicity of isoflucypram has been well investigated in a standard OECD 416 guideline rat two-generation study. It is noted that there were no TSH or T4 measurements and no investigations of nipple retention. These limitations have no impact on the validity of the study as thryoid hormones were subsequently investigated

in mechanistic studies (see section B.6.8.2) and the lack of nipple retention meassurements does not constitute a data gap as two negative *in vivo* mechanistic studies on the potential oestrogenic and androgenic activity of isoflucypram are available (see section B.6.8.3).

Study	Two-Generation Reproductive Toxicity
Reference	[redacted] (2018)
Date performed	04/06/2015-09/03/16
Test facility	[redacted]
Report reference	Study number Sponsor reference TXLNN124
Guideline(s)	OECD TG 416
Deviations from the guideline	None
GLP	Yes. Signed QA and GLP certificates provided
Test material	Isoflucypram; Batch 2013-006492; purity 94.2% w/w%
Study acceptable	Yes

Methods

The potential effects of isoflucypram on sexual function and fertility have been investigated in a standard two-generation study in the rat at dietary concentrations of 0, 150/75 (equivalent to 11.27 – 14.62 mg/kg bw/d), 450/225 (equivalent to 34.1 – 44.5 mg/kg bw/d) and 1200/600 ppm (equivalent to 92.9 – 140.4 mg/kg bw/d). The dietary concentrations of 150, 450 and 1200 ppm were adjusted to 75, 225 and 600 ppm in females during lactation to ensure the achieved dose levels/intakes during this period were equivalent to those in the pre-mating phase. In this study, F0 parental animals (28/sex/dose group) were administered isoflucypram via the diet for a 10 week pre-mating period, throughout mating, gestation, and lactation. Litters were standardised on PND 4 to 4 males/females per litter. Selected F1 pups (24/sex/dose group) were retained post weaning, and mated to produce the F2 generation. Adult males were sacrificed after 17 weeks and young females on Post Partum Day (PPD) 28. One male and female pups from each litter were sacrificed on post-natal day (PND) 21. The stability and homogeneity of the test substance in the diet was acceptable. Calculated achieved intakes were:

Table B.6.6-1: Achieved intakes of isoflucypram

Phase	**Generation**	**Dietary concentration isoflucypram in ppm**					
		Males			**Females[1]**		
		150	**450**	**1200**	**150/75**	**450/225**	**1200/600**
Pre-mating (mg/kg bw/day)	F0	11.27	34.1	94.4	13.03	40.8	140.4
	F1	13.91	41.6	108.6	14.62	44.5	112.5
Gestation (mg/kg bw/day	F0				11.69	35.9	94.7
	F1				12.94	38.4	102.5
Lactation (mg/kg bw/day)	F0				11.49	35.8	94.5
	F1				11.28	34.1	92.9

[1]Dietary concentrations were reduced during gestation and lactation to 75, 225, and 600 ppm in order to maintain a constant achieved intake.

A method of analysis for isoflucypram in the diet at the different concentrations tested is available, but has not been submitted. This should be considered during the peer-review process.

Results

The doses were selected on the basis of a preliminary one-generation range-finding dietary study in which males and females were administered isoflucypram at concentrations of 0, 100, 300, and 1000 ppm for four weeks before pairing, during pairing and during gestation. There were no effects seen with regard to clinical signs, body weight or body weight gain, estrus cyclicity, pre-mating interval, fertility, fecundity in the parents, and no effects observed on body weight gain or sexual maturation of the offspring. The only treatment-related effects were seen at 1000 ppm; an increase in relative liver weight in F0 males and F1 males and females, and at 300 ppm, an increase in relative liver weight in F1 males.

Parental and offspring toxicity

In the full 2-generation study, there were no effects of treatment on clinical signs or body weight, although food consumption was statistically significantly decreased (around 15%) at the end of the pre-mating phase in F1 females at the top dose. In relation to general toxicity, liver, thyroid, thymus and kidney weight changes were

observed in one or more generations in adult animals or in pups sacrificed on PND 21, in general from the mid-dose of 450/225 ppm. These changes are reported and discussed below.

Table B.6.6-2: Mean terminal body weight and absolute and relative organ weights in male rats

Gen	Phase	Parameter	Isoflucypram dietary conc. in ppm			
			0	**150**	**450**	**1200**
F0	Week 17	Terminal body wt, g	438	444	439	430
		Liver wt, g (% change)	14.45	14.45	15.01	15.94** **(9%)**
		Liver wt, relative to body wt (% change)	3.30	3.25	3.42	3.70** **(11%)**
		Thyroid wt, g (% change)	0.017	0.018	0.020* **(18%)**	0.019* **(12%)**
		Thyroid wt, relative to body wt (% change)	0.0038	0.0041	0.0045* **(18%)**	0.0045** **(18%)**
		Thymus wt, g	0.285	0.276	0.290	0.286
		Thymus wt relative to body wt	0.0650	0.0623	0.0663	0.0666
		Kidney wt (g)	2.85	2.6	2.5	2.9
		Kidney wt relative to body wt (% chnage)	0.647	0.585	0.579	0.68* **(5%)**
F1	PND 21	Terminal body wt, g	48.3	48.4	49.2	49.3
		Liver wt, g (% change)	2.174	2.272	2.408* **(12%)**	2.551** **(17%)**
		Liver wt, relateive to body wt	4.513	4.671	4.874** **(8%)**	5.157** **(15%)**
		Thyroid wt, g	0.0056	0.0056	0.0058	0.0058
		Thyroid wt, relateive to body wt	0.0116	0.0117	0.0117	0.0118
		Thymus wt, g	0.202	0.204	0.208	0.206
		Thymus wt relative to body wt	0.419	0.423	0.424	0.418
	Week 17	Terminal body wt, g	471	473	461	454
		Liver wt, g (% change)	16.14	15.43	16.18	17.58* **(7%)**
		Liver wt, relateive to body wt (% change)	3.44	3.27	3.51	3.87** **(13%)**
		Thyroid wt, g	0.020	0.020	0.021	0.021
		Thyroid wt relative to body wt	0.0043	0.0043	0.0045	0.0045
		Thymus wt, g (% change)	0.350	0.336	0.337	0.293* **(16%)**
		Thymus wt relative to body wt (% change)	0.0741	0.0716	0.0729	0.0645 **(14%)**
		Kidney wt (g)	2.78	2.77	2.74	2.79
		Kidney wt relative to body wt	0.591	0.588	0.595	0.614
F2	PND 21	Terminal body wt, g	49.9	50.3	49.9	48.2
		Liver wt, g (% change)	2.245	2.410	2.454* **(9%)**	2.473* **(10%)**
		Liver wt, relative to body wt (% change)	4.498	4.787* **(6%)**	4.889* **(9%)**	5.127** **(14%)**
		Thyroid wt, g	0.0060	0.0061	0.0068	0.0061
		Thyroid wt, relateive to body wt	0.0122	0.0121	0.0137	0.0127
		Thymus wt, g	0.211	0.217	0.211	0.203

Gen	Phase	Parameter	Isoflucypram dietary conc. in ppm			
			0	**150**	**450**	**1200**
		Thymus wt relative to body wt	0.421	0.434	0.425	0.420

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$
PND – Post Natal Day
PPD – Post Partum Day

Table B.6.6-3: Mean terminal body weight and absolute and relative organ weights in female rats

Gen	Phase	Parameter	Isoflucypram dietary conc. in ppm			
			0	**150/75**	**450/225**	**1200/600**
F0	PPD 28	Terminal body wt, g	250	251	249	245
		Liver wt, g (% change)	10.46	10.68	11.32** **(9%)**	12.33** **(18%)**
		Liver wt, relateive to body wt (% change)	4.19	4.27	4.55** **(9%)**	5.04** **(20%)**
		Thyroid wt, g	0.017	0.016	0.016	0.017
		Thyroid wt relative to body wt	0.0067	0.0065	0.0063	0.0068
		Thymus wt, g (% change)	0.257	0.250	0.243	0.222* **(14%)**
		Thymus wt relative to body wt (% change)	0.1030	0.1002	0.0971	0.0906* **(12%)**
F1	PND 21	Terminal body wt, g	46.7	46.8	47.2	48.5
		Liver wt, g (% change)	2.031	2.210* **(9%)**	2.333** **(15%)**	2.571** **(27%)**
		Liver wt, relateive to body wt (% change)	4.417	4.703* **(7%)**	4.936** **(11%)**	5.293** **(20%)**
		Thyroid wt, g	0.0058	0.0057	0.0059	0.0060
		Thyroid wt relative to body wt	0.0124	0.0123	0.0126	0.0124
		Thymus wt, g	0.210	0.217	0.215	0.217
		Thymus wt relative to body wt	0.450	0.464	0.453	0.447
	PPD 28	Terminal body wt, g	268	270	265	262
		Liver wt, g	11.76	11.97	12.60*	13.02**
		Liver wt, relateive to body wt (% change)	4.39	4.44	4.76** **(8%)**	4.97** **(13%)**
		Thyroid wt, g	0.017	0.016	0.017	0.017
		Thyroid wt relative to body wt	0.0062	0.0059	0.0062	0.0066
		Thymus wt, g	0.274	0.281	0.282	0.254
		Thymus wt relative to body wt	0.102	0.104	0.107	0.097
F2	PND 21	Terminal body wt, g	47.2	48.5	47.3	46.4
		Liver wt, g (% change)	2.181	2.401* **(10%)**	2.314* **(6%)**	2.382* **(9%)**
		Liver wt, relative to body wt (% change)	4.617	4.933* **(7%)**	4.873* **(6%)**	5.126** **(11%)**
		Thyroid wt, g	0.0060	0.0064	0.0064	0.0062
		Thyroid wt relative to body wt	0.0128	0.0134	0.0136	0.0135
		Thymus wt, g	0.199	0.219	0.214	0.212
		Thymus wt relative to body wt	0.421	0.454	0.451	0.455

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

Liver: In F0 parental males, sacrificed after 17 weeks, statistically significantly increased absolute (by 9%) liver weights were observed at the top dose of 1200 ppm only. In F1 parental males, statistically significantly increased absolute (by 7%) and relative liver weights (by 13%) were observed at the top dose of 1200 ppm only. In F1 male pups sacrificed on PND 21, absolute and relative liver weights were stastically significantly increased at doses of 450 ppm and above (by 12% a/8% r and 17% a/15% r at 450 and 1200 ppm respectively). In F2 male pups sacrificed on PND 21, absolute liver weights were stastically significantly increased at doses of 450 ppm and above (by 9-10%) and relative liver weights were statistically significantly at all dose levels (by 6%, 9% and 14% at 150, 450 and 1200 ppm respectively).

In F0 parental females, sacrificed on post partum day (PPD) 28, absolute and relative liver weights were statistically significantly increased at doses of 450 ppm and above (by 9% a/9% r and 18% a/20% r at 450 and 1200 ppm respectively). In F1 female pups sacrificed on PND 21, absolute and relative liver weights were statistically significantly increased from the lowest dose (by 9% a/7% r, 15% a/11% r and 27% a/20% r at 150, 450 and 1200 ppm respectively). In F2 female pups sacrificed on PND 21, absolute and relative liver weights were also statistically significantly increased at all doses tested (by 10% a/7% r, 6% a/6% r and 9% a/11% r at 150, 450 and 1200 ppm respectively).

Overall, adverse (>15%) increased liver weights were seen at the top dose in adult females and young males and females in both generations. However, in F1 female PND21 pups, an adverse increased liver weight was also seen at the mid-dose of 450 ppm.

Thyroid: Statistically significant increases in thyroid weights were only seen in F0 adult males from the mid-dose of 450 ppm (by 18% for relative weight at both 450 and 1200 ppm). Thyroid weights were not increased in females, in the young or in the second generation. However, despite this inconsistency, as it is well established that the thyroid is a target organ of toxicity in rats in the repeated dose toxicity studies, this finding is considered by the RMS to be treatment-related and adverse.

Kidney: A statistically significant increase (by 5% for relative weight) in kidney weight was seen only in F0 parental males at the top dose. Despite the small magnitude of the increase and the inconsistency of the finding across generations and life stages, the RMS considers the effect treatment-related and adverse as similar effects on the kidney were seen in the male rat in the repeated dose toxicity studies. It is most likely that these changes were secondary to α-2μ globulin accumulation (see section B.6.3), a mechanism which is not relevant to humans.

Thymus: A statistically significant decrease in absolute and relative thymus weight was seen at the top dose only in F1 parental males (by 16% for absolute weight) and in F0 PPD28 females (by 14% for absolute weight). Given the inconsistency of the finding across generations and life stages, the lack of associated histopathology and the absence of similar effects in the repeated dose toxicity studies, the RMS concludes that the effect is most likely a chance finding unrelated to treatment.

It is notable that no adverse histopathological changes were observed in any organs and tissues investigated up to the top dose of 1200/600 ppm, in either male or female parental animals, pups and generations.

Clinical chemistry investigations in parental animals found changes in some parameters associated with liver toxicity, at doses of 450 ppm and above (* = statistical significance). Bile acids were decreased at 450 (m*38%/f 38%) and 1200 ppm (m* 45%/f 42%); ALP was decreased in high dose F0 males only (m*26%); cholesterol levels were increased in high dose F0 (m 12%/f* 46%),) and F1 animals (m* 18%/f 22%); triglycerides were increased in high dose F0 (m 12%/f*59%) and F1 animals (m* 15%/f* 29%) and mid-dose F1 animals (m* 18%/f 22%); and creatinine was increased in F1 males at 450 (m* 6%) and 1200 ppm (m* 12%). In addition there were statistically significant increases in calcium and total protein in both F0 and F1 males and females at the top dose. The decreases in ALP and bile acids were not considered toxicologically relevant. No treatment-related clinical chemistry changes were observed at 150 ppm, the lowest dose tested.

Table B.6.6-4: Effects of isoflucypram on selected clinical-chemistry parameters (bile acids, ALP, cholesterol, triglycerides; creatinine) in parental animals (F0 and F1).

		Males				Females			
	Parameter	**Control**	**Low Dose**	**Mid Dose**	**High Dose**	**Control**	**Low Dose**	**Mid Dose**	**High Dose**
F0 (Week 9)	ALP (U/L)	100 ± 19.5	89 ± 26.8 (-11%)	83 ± 18.1 (-17%)	74** ± 15.4 (-26%)	41 ± 10.2	45 ± 16.1 (+9.8%)	44 ± 14.1 (+7.3%)	36 ± 12.0 (-12.2%)
	Bile Acid (μmol/L)	41.4 ± 13.31	34.9 ± 12.30	26.2** ± 9.62	25.6** ± 8.32	39.4 ± 25.99	46.4 ± 25.01	27.8 ± 20.28	24.6 ± 11.87

			(-15.7%)	(-36.7%)	(-38.2%)		(+17.8%)	(-29.4%)	(-37.6%)
	Creatinine (µmol/L)	38 ± 4.4	37 ± 2.6 (-2.6%)	41 ± 4.6 (+7.9%)	40 ± 6.6 (+5.3%)	44 ± 5.2	42 ± 5.5 (-4.5%)	44 ± 4.7 (no change)	39 ± 2.8 (-11.4%)
	Cholesterol (mmol/L)	2.03 ± 0.222	2.07 ± 0.308 (+2%)	2.09 ± 0.275 (+3%)	**2.27** ± 0.298 (+11.8%)	1.75 ± 0.381	1.93 ± 0.635 (+10.3%)	1.97 ± 0.344 (+12.6%)	**2.56**** ± 0.402 (+46.3%)
	Triglyceride (mmol/L)	1.04 ± 0.398	1.15 ± 0.516 (+10.6%)	1.11 ± 0.363 (+6.7%)	**1.16** ± 0.207 (+11.5%)	0.58 ± 0.302	0.61 ± 0.247 (+5.2%)	0.70 ± 0.407 (+20.7%)	**0.92*** ± 0.393 (+58.6%)
	Calcium	2.67 ± 0.063	2.66 ± 0.068 (- 0.4%)	2.69 ± 0.068 (+ 0.7%)	**2.79*** ± 0.063 (+4%)	2.67 ± 0.076	2.68 ± 0.067 (+0.4%)	2.70 ± 0.058 (+1%)	**2.79**** ± 0.098 (+4.5%)
	Total protein	66 ± 0.9	68* ± 1.7 (+3%)	68* ± 1.5 (+3%)	**69**** ± 1.9 (+5%)	68 ± 3.1	67 ± 3.5 (-1%)	69 ± 3.0 (+1%)	**73**** ± 3.4 (+7%)
F1 (Week 9)	ALP (U/L)	83 ± 12.7	87 ± 17.1 (+4.8%)	90 ± 17.5 (+8.4%)	73 ± 19.4 (-12 %)	48 ± 7.6	41 ± 7.5 (-14.6%)	41 ± 10.9 (-14.6%)	41 ± 15.4 (-14.6%)
	Bile Acid (µmol/L)	38.2 ± 28.84	38.3 ± 8.15 (no change)	43.4 ± 42.85 (+13.6%)	21.0 ± 7.95 (-45%)	30.3 ± 37.63	27.0 ± 26.60 (-10.9%)	18.3 ± 13.21 (-39.6%)	17.5 ± 15.82 (-42.2%)
	Creatinine (µmol/L)	34 ± 2.1	32 ± 2.1 (-5.9%)	38** ± 2.3 (+11.8)	36** ± 3.1 (+5.9%)	38 ± 3.4	40 ± 3.0 (+5.3%)	36 ± 2.2 (-5.3%)	37 ± 4.1 (-2.6%)
	Cholesterol (mmol/L)	1.72 ± 0.243	1.61 ± 0.183 (-6%)	**2.03**** ± 0.235 (+18%)	**1.97**** ± 0.189 (+14.5%)	1.60 ± 0.456	1.66 ± 0.581 (+3.8%)	1.95 ± 0.258 (+21.9%)	**2.07*** ± 0.381 (+29.4%)
	Triglyceride (mmol/L)	0.75 ± 0.325	0.94 ± 0.378 (+25%)	0.75 ± 0.232 (no change)	**0.84** ± 0.314 (+12%)	0.44 ± 0.148	0.44 ± 0.172 (no change)	0.62 ± 0.295 (+40.9%)	**0.54** ± 0.240 (+22.7%)
	Calcium	2.54 ± 0.07	2.56 ± 0.065 (+ 0.7%)	2.59 ± 0.081 (+ 2%)	**2.62*** ± 0.07* (+3%)	2.54 ± 0.088	2.59 ± 0.074 (+2%)	2.56 ± 0.072 (+0.8%)	2.58 ± 0.064 (+1.5%)
	Total protein	66 ± 2.2	67 ± 2.6 (+1.5%)	68 ± 1.5 (+3%)	**69*** ± 3.2 (+5%)	68 ± 2.8	70 ± 3.2 (+3%)	70 ± 2.9 (+3%)	71 ± 2.3 (+4%)

Data obtained from Table 15 (F0) and Table 50 (F1). For each data entry N = 10. Values in parentheses indicate % change (rounded-up) compared to controls. *: $p<0.05$, **: $p<0.01$.

Reproductive Toxicity

Oestrous cyclicity, pre-coital interval, mating performance and fertility were not affected by treatment. There were no effects on reproductive performance (litter size, offspring survival and development), ovarian primordial follicle counts, sperm parameters, sex ratio, AGD and weights, macroscopic and microscopic appearance of reproductive organs. There were no clear effects on gestation index, but a small decrease in gestation length was seen only in the F1 generation at the top dose. In the absence of effects on any other reproductive parameters, it is most likely this effect was the consequence of the liver toxicity observed in these animals at this dose rather than an expression of specific reproductive toxicity of isoflucypram.

Table B.6.6-5: Oestrous Cyclicity, Pre-Coital Interval, Mating Performance, Fertility, Gestation Length

			Females				Males			
	Parameter		**Control**	**Low Dose**	**Mid Dose**	**High Dose**	**Control**	**Low Dose**	**Mid Dose**	**High Dose**
F0	Oestrous Cyclicity	Regular[A]	27/28 (96%)	25/28 (89%)	25/27 (93%)	25/28 (89%)				
		Irregular[B]	1/28 (4%)	2/28 (7%)	1/27 (4%)	2/28 (7%)				
		Acyclic[C]	0	1/28 (4%)	1/27 (4%)	1/28 (4%)				
	Pre-Coital Interval	1-4 days	28/28 (100%)	28/28 (100%)	26/26 (100%)[D]	27/28 (96%)				
		5-8 days	0	0	0	1/28 (4%)				
	Gestation length	22 days	25%	32%	25%	14%				
		22.5 days	25%	18%	38%	39%				
		23 days	50%	50%	38%	46%				
	Mating (N =28)		100%	100%	100% (N = 27)	100%	100%	100%	100% (N = 27)	100%
	Conception Rate		100%	100%	96%	100%	100%	100%	96%	100%
	Fertility Index		100%	100%	96%	100%	100%	100%	96%	100%
F1	Oestrous Cyclicity	Regular[A]	24/24 (100%)	24/24 (100%)	22/24 (92%)	24/24 (100%)				
		Irregular[B]	0/24	0/24	0/24	0/24				
		Acyclic[C]	0/24	0/24	2/24 (8%)	0/24				
	Pre-Coital Interval (days)	1-4	22/24 (92%)	24/24 (100%)	24/24 (100%)	24/24 (100%)				
		5-8, 9-12	0	0	0	0				
		13-14	2/24 (8%)	0	0	0				
	Gestation length	22 days	17%	9%	33%	29%				
		22.5 days	48%	65%	42%	58%				
		23 days	35%	26%	25%	13%				
	Mating (N = 24)		100%	100%	100%	100%	100%	100%	100%	100%
	Conception Rate		96%	96%	100%	100%	96%	96%	100%	100%
	Fertility Index		96%	96%	100%	100%	96%	96%	100%	100%

Data obtained from Tables 16-18 for F0 and Tables 52-54 for F1. A: regular cycle data combined for 4 day and 4/5 day lengths (no cycles of 5 days were observed); B: Irregular cycles: at least one cycle of 2, 3 or 6 – 10 days; C: Acyclic: At least 10 days without oestrous. D: One female excluded as date of mating not established. Values in parentheses indicate % change (data have been rounded up).

Table B.6.6-6: Reproductive performance (litter size, offspring survival), sex ratio.

Parameter	**Control**	**Low Dose**	**Mid Dose**	**High Dose**
F1 Generation				
Number born live	305	295	292	304
Number born dead[A]	2	1	0	2
Sex ratio day 1 (% M)[B]	53 ± 14.4	45.1* ± 12.5	49.8 ± 13.7	52.2 ±14.2
# Deaths days 1- 4[A]	1	2	1	1
# Deaths days 5-21[A]	6	1	1 (+ 1KFHR)	1
Mean litter size				
Day 1	10.8 ± 2.4	10.9 ± 2.2	11.7 ± 2.5	10.8 ± 2.6
Day 4 (before cull)	10.8 ± 2.4	10.8 ± 2.2	11.6 ± 2.5	10.8 ± 2.5
Day 4 (after cull)	7.8 ±0.5	7.9 ± 0.4	7.9 ±0.6	7.8 ± 0.7
Day 7	7.8 ± 0.6	7.9 ± 0.4	7.9 ±0.6	7.7 ± 0.7
Day 14	7.6 ± 0.8	7.9 ± 0.4	7.8 ±0.6	7.7 ± 0.7

Day 21	7.6 ± 0.8	7.9 ± 0.4	7.8 ±0.6	7.7 ± 0.7
Birth Index				
Live birth Index (%)	98.9	100	100	99.2
Viability Index (%)	100	99.6	99.7	99.7
Lactation Index (%)	97.2	99.5	99.0	99.6
F2 Generation				
Number born live	285	272	296	293
Number born dead[A]	1	2	1 (+ 1 partially cannablized)	2
Sex ratio day 1 (% M)	50.8 ± 13.2	46.2 ± 14.1	51.2 ± 15.7	46.2 16.7
# Deaths days 1- 4[A]	0	1	0	0 (+ 1 KFHR)
# Deaths days 5-21[A]	1 (+ 1KFHR)	0	1 (+ 1 KFHR)	1
Mean litter size				
Day 1	12.3 ± 2.2	11.7 ± 2.4	12.3 ±2.0	12.0 ± 2.0
Day 4 (before cull)	12.3 ± 2.2	11.6 ± 2.3	12.3 ± 2.0	12.0 ± 2.0
Day 4 (after cull)	8.0 ± 0.2	7.9 ± 0.5	7.9 ± 0.4	8.0 ± 0.0
Day 7	7.9 ± 0.3	7.9 ± 0.5	7.9 ± 0.4	8.0 ± 0.0
Day 14	7.9 ± 0.3	7.9 ± 0.5	7.8 ± 0.5	8.0 ± 0.2
Day 21	7.9 ± 0.3	7.9 ± 0.5	7.8 ± 0.5	8.0 ± 0.2
Birth Index				
Live birth Index (%)	99.7	98.8	99.4	98.7
Viability Index (%)	100	99.4	100	99.6
Lactation Index (%)	98.9	100	99	99.5

Data taken from Tables 20-22 and Appendices 11-12 for F1 and Tables 56-58 and Appendices 38-39 for F2. A: Includes pups indicated as missing. B: May include offspring that died prior to the designated Day 1 of age. Unsexed offspring missing prior to Day 1 are not included. KFHR: killed for humane reasons. * p≤0.05

Table B.6.6-7: Anogenital Distance.

Sex	Control	Low Dose	Mid Dose	High Dose
F2 Generation				
Male	4.6 ± 0.3 (N=23)	4.7± 0.4 (N=23)	4.6 ± 0.3 (N=24)	4.7 ± 0.4 (N=24)
Female	2.7 ± 0.2 (N=23)	2.7 ± 0.2 (N=23)	2.7 ± 0.2 (N=24)	2.6 ± 0.2 (N=24)

No evaluation performed on F1 generation

Table B.6.6-8: Sperm parameters

Sperm Analysis		Control	Low Dose	Mid Dose	High Dose
F0 Generation Males					
N		28	28	28	28
Sperm Motility	% Motile	90 ± 7	91 ± 8	90 ± 9	91 ± 9
	% Progressive	52 ± 9	52 ± 10	54 ± 9	54 ± 9
Sperm Counts (millions/g)	Testis	148 ± 28	NI	NI	145 ± 29
	Epididymis	780 ± 183	NI	NI	838 ± 247
Sperm Morphology (%)	Normal	96.8 ± 1.9	NI	NI	96.5 ± 2.3
	Abnormal	3.2 ± 1.9	NI	NI	3.5 ± 2.3
F1 Generation Males					
N		23	24	24	24
Sperm Motility	% Motile	91 ± 5	89 ± 7	90 ± 6	90 ± 7
	% Progressive	52 ± 10	46 ± 11	50 ± 7	46 ± 12
Sperm Counts (sperm/g)	Testis	113 ± 30	NI	NI	113 ± 17
	Epididymis	693 ± 161	NI	NI	621 ± 171
Sperm Morphology (%)	Normal	96.6 ± 1.6	NI	NI	96.4 ± 1.8
	Abnormal	3.4 ± 1.6	NI	NI	3.6 ± 1.8

NI: not investigated.

Table B.6.6-9: Landmark Development (F1)

	Balano Preputial Separation	Vaginal Opening

Parameter	N	Age at completion (days)	Body Weight (g) at Completion	N	Age at completion (days)	Body Weight (g) at Completion
Control	24	46 ± 3.4	196 ± 23.9	24	33 ± 2.6	105 ± 13.3
Low Dose	24	46 ± 3.1	192 ± 19.4	24	33 ± 2.3	103 ± 13.6
Mid dose	24	47 ± 2.2	196 ± 18.6	23	33 ± 2.3	104 ± 14.6
High Dose	24	48 ± 4.0	202 ± 22.7	24	38** ± 2.9	124** ± 11.8

** p≤0.01.

Table B.6.6-10 Male reproductive organs

Parameter			**Control**	**Low Dose**	**Mid Dose**	**High Dose**
	F0 Males					
N			28	28	28	28
	Terminal Bodyweight (g)		438 ± 37	444 ± 38	439 ± 34	430 ±29
Weight relative to Bodyweight	Prostate		0.175 ± 0.033	0.179 ± 0.028	0.185 ± 0.028	0.190 ± 0.036
	Seminal Vesicles		0.366 ± 0.055	0.373 ± 0.063	0.354 ± 0.053	0.361 ± 0.067
	Epididymides		0.311 ± 0.030	0.298 ± 0.041	0.313 ± 0.033	0.324 ± 0.042
	Testes		0.867 ± 0.083	0.853 ± 0.093	0.864 ± 0.057	0.887 ± 0.080
Macroscopic appearance	Prostate		NA	NA	NA	NA
	Seminal Vesicles		NA	NA	NA	NA
	Epididymides		NA	NA	NA	NA
	Testes		NA	NA	NA	Right testis small: 1/28
Microscopic findings	Prostate	Erosion, epithelial	Minimal: 1/28	NA	NA	NA
		Inflammation	Minimal: 1/28	NA	NA	NA
		Lymphoid aggregates	Minimal: 2/28 Slight: 1/28	NA	NA	Minimal: 1/28
	Seminal Vesicles and coagulating glands		NA	NA	NA	NA
	Right Epididymis	Epithelial vacuolation	Minimal: 5/28	NA	NA	Minimal: 4/28
		Inflammatory cell perivascular	NA	NA	NA	Minimal: 1/28
	Right Testes	Seminiferous tubular atrophy	Minimal: 1/28	NA	NA	Minimal: 1/28
		Seminiferous tubular degeneration	Minimal: 3/28	NA	NA	Minimal: 1/28

Table B.6.6-11: Male reproductive organs (continued)

Parameter		**Control**	**Low Dose**	**Mid Dose**	**High Dose**
	F1 Males (on Day 21 of age)				
N		25	27	25	28
	Terminal Bodyweight (g)	48.3 ± 4.0	48.4 ± 4.8	49.2 ± 5.1	49.3 ± 5.0
	No male reproductive organs taken				
	F1 Males (after 17 weeks of treatment)				
N		23	24	24	24
	Terminal Bodyweight (g)	471 ± 41	473 ± 41	461 ± 41	454 ± 42

Weight relative to Bodyweight	Prostate		0.181 ± 0.039	0.195 ± 0.048	0.188 ± 0.040	0.182 ± 0.040
	Seminal Vesicles		0.330 ± 0.062	0.328 ± 0.061	0.344 ± 0.061	0.351 ± 0.078
	Epididymides		0.331 ± 0.045[A]	0.326 ± 0.046[B]	0.335 ± 0.029	0.331 ± 0.035[B]
	Testes		0.872 ± 0.108	0.861 ± 0.097	0.890 ± 0.096	0.907 ± 0.098
Macroscopic appearance	Prostate		NA	NA	NA	NA
	Seminal Vesicles		NA	NA	NA	NA
	Epididymides		NA	NA	NA	NA
	Testes		NA	NA	NA	NA
Microscopic findings	Prostate	Inflammation	NA	NA	NA	Minimal: 1/24
		Lymphoid aggregates	NA	NA	NA	Minimal: 2/2
	Seminal vesicles		NA	NA	NA	NA
	Right Epididymis	Degenerate spermatogenic cells in duct(s)	NA	NA	NA	Minimal: 1/24
		Inflammatory cell infiltrate perivascular	NA	NA	NA	Minimal: 1/24
	Right testis	Seminiferous tubular atrophy	Minimal: 1/23	NA	NA	Minimal: 1/24 Slight: 1/24

NA: not affected; NI: not investigated. A: N =22/23; B = 23/24

Table B.6.6-12: Female reproductive organs

Parameter			**Control**	**Low Dose**	**Mid Dose**	**High Dose**
	F0 Females					
N			28	27	25	28
	Terminal Bodyweight (g)		250 ± 18	251 ± 20	249 ± 20	245 ± 13
Weight relative to Bodyweight	Ovaries		0.0466 ± 0.0080	0.0432 ± 0.0088	0.0460 ± 0.0084	0.0460 ± 0.0081
	Uterus/Cervix/Oviduct		0.286 ± 0.102	0.308 ± 0.093	0.266 ± 0.070	0.298 ± 0.091
Macroscopic appearance	Uterus: Fluid distension		4/28	4/28	2/25	1/28
Microscopic findings	Left & Right ovary and oviduct		NA	NA	NA	NA
	Uterus	Glandular dilatation	NA	NA	NA	Minimal: 1/28
		Luminal Dilatation (present)	3/28	1/3	NI	4/28
	Uterine Cervix		NA	NA	NI	NA
	Vagina	Dioestrous (present)	4/28	0/3	NI	7/28
		Metoestrous (present)	7/28	1/3	NI	4/28
		Oestrous (present)	14/28	2/3	NI	13/28
		Proestrous (present)	3/28	0/3	NI	4/28
	F1 Females (age 21 Days)					
N			27	27	24	28
	Terminal Bodyweight (g)		46.7± 3.4	46.8 ± 4.2	47.2 ± 5.1	48.5 ± 4.2
Weight relative to Bodyweight	Uterus		0.093 ± 0.018	0.092 ± 0.020	0.095 ± 0.018	0.100 ± 0.021
No macroscopic observations; no microscopic assessments						

NA: not affected; NI: not investigated.

Table B.6.6-13: Female reproductive organs (continued)

Parameter			Control	Low Dose	Mid Dose	High Dose
	F1 Females (28 days post-partum)					
N			23	23	24	24
	Terminal Bodyweight (g)		268 ± 21	270 ± 17	265 ± 21	262 ± 13
Weight relative to Bodyweight	Ovaries		0.0465 ± 0.0081	0.0441 ± 0.0063	0.0448 ± 0.0072	0.0451 ± 0.0081
	Uterus/Cervix/Oviduct		0.288 ± 0.093	0.261 ± 0.068	0.268 ± 0.078	0.280 ± 0.106
Macroscopic appearance	Uterine cervix (masses)		NA	NA	NA	1/24
	Uterus:	Fluid distension	5/23	2/23	5/24	5/24
		Pregnancy comments	NA	1/23	NA	NA
	Left ovary: Periovarian sac distension		NA	NA	NA	1/24
	Right ovary: Periovarian sac distension		NA	1/23	NA	NA
Microscopic findings	Left & Right ovary and oviduct		NA	NA	NA	NA
	Uterus		NA	NA	NA	NA
	Uterine Cervix: Squamos Epithelial cyst(s)		NA	NA	NA	1/24
	Vagina	Dioestrous (present)	6/23	NI	1/2	9/24
		Metoestrous (present)	3/23	NI	0/2	3/24
		Oestrous (present)	11/23	NI	1/2	7/24
		Proestrous (present)	3/23	NI	0/2	5/24
	F2 Females (21 days of age)					
N			23	22	24	24
	Terminal Bodyweight (g)		47.2 ±4.5	48.5 ± 3.9	47.3 ± 4.6	46.4 ± 3.2
Weight relative to Bodyweight	Uterus		0.108 ± 0.040	0.103 ± 0.034	0.104 ± 0.021	0.115 ± 0.047
No macroscopic observations; no microscopic assessments						

NA: not affected; NI: not investigated.

The mean age at vaginal opening for F1 females in the 1200/600 ppm (94.5 mg/kg/d) group occurred five days later than in controls. The body weight of these F1 females was also significantly increased. In the presence of an increase in body weight (usually delayed vaginal opening is associated with reduced body weight), it is difficult to establish the toxicological significance of the observed delay in vaginal opening. However, in the absence of any effects on other developmental landmarks and on ano-genital distance (AGD) and considering that these females went on to mate successfully and produce the F2 generation, the RMS concludes that it is most likely this observation was a chance finding. In addition, no effects on vaginal opening were seen up to the high gavage dose of 400 mg/kg bw/d for 20 days in a modified rat uterotrophic assay in immature animals ([redacted] 2011; see section B.6.8.3) which included specific investigations of vaginal opening.

Table B.6.6-14: Group mean age and body weight at vaginal opening in F1 female pups

Sex	Parameter	Isoflucypram dietary concentration in ppm			
		0	**150 / 75**	**450 / 225**	**1200 / 600**
Females	Age, days	33	33	33	38**
	Body weight, g	105	103	104	124**

Significant at * $p \leq 0.05$; ** $p \leq 0.01$

At the top dose of 1200/600 ppm (93-140 mg/kg/d), a higher litter incidence of bilateral or unilateral dilated renal pelvis (9/28 litters versus 5/28 control litters) was observed among F1 offspring sacrificed on PND21. It is noted that with the exception of a minor increase in relative kidney weights in F0 parental males, no kidney weight changes or treatment-related macro- or histopathological changes were observed, in either generation. The absence of abnormal kidney pathology in F1 parental animals gives reassurance that the dilated renal pelvis observed in F1 PND 21 pups did not lead to any macroscopic or microscopic changes in adult animals. Dilated renal pelvis was observed in the rat developmental toxicity study but only at the high dose of 625 mg/kg bw/d (and not at the low- and mid-dose of 25 and 125 mg/kg bw/d, respectively). Therefore, the RMS concludes that this finding observed at the relatively low dose of 94.5 mg/kg bw/d is either a chance finding or is of minimal/no toxicological significance.

Conclusion

The potential of isoflucypram to adversely affect reproduction has been well investigated in a standard 2-generation dietary study, conducted in rats.

There were no effects of treatment on mating behaviour, fertility, litter size, oestrus cycle, ovarian primordial follicle counts, offspring survival and development, sex ratio, AGD, sexual maturity and gestation up to the top dose of 1200/600 ppm (93 – 104 mg/kg bw/d), a dose at which parental and offspring toxicity occurred. In addition, examination of the reproductive organs did not reveal any treatment-related changes. Specific investigations of the spermatogenic cycle did not find any cell or stage-specific abnormalities at any dose level. Therefore a NOAEL for reproductive toxicity of 93-104 mg/kg bw/d (highest dose tested) can be identified from this study.

In relation to general toxicity in parental animals and offspring, liver and thyroid weights (with no associated histopathology) were adversely increased from the mid dose of 450/225 ppm (equivalent to 34.1-40.8 mg/kg/day) in adults and pups in both generations. Clinical-chemistry parameters associated with liver effects were also affected from the mid dose in parental animals of both generations. In addition, food consumption was decreased in F1 adult females at the top dose. There were no adverse effects in parental animals and offspring at the lowest dose tested of 150/75 ppm (11-14 and mg/kg/d). Therefore a NOAEL of 11-14 mg/kg bw/d can be identified for parental and offspring toxicity from this study.

(*[redacted] 2018)*

B.6.6.2. Developmental toxicity studies

The developmental toxicity of isoflucypram has been well investigated in standard OECD 414 guideline studies, one conducted in rats and the other in rabbits.

Rats

Study	Developmental Toxicity
Reference	[redacted] (2017b)
Test facility	[redacted]
Report reference	Study number Sponsor reference SA 14192
Guideline(s)	OECD TG 414 (2001)
Deviations from the guideline	None
GLP	Yes. Signed QA and GLP certificates provided
Test material	Isoflucypram technical; Batch: 2013-006492, Purity 94.2% w/w,
Study acceptable	Yes

Methods

Isoflucypram was administered to groups of female time-mated rats (Sprague-Dawley, 23/dose) by oral gavage on days 6-20 of gestation at doses of 0, 25, 125, and 625 mg/kg bw/d, in aqueous 0.5% methylcellulose 400. The test substance was stable in the chosen vehicle. Plasma levels of isoflucypram and of two metabolites (BCS-CX99798 and BCS-CX99799) were measured in 5 dams from each group at necropsy.

The doses employed were selected based on the results of a range-finding study in which pregnant rats were administered isoflucypram from GD6 through GD 20 at doses of 0, 70, 250, or 700 mg/kg bw/d. A fully validated method of analysis for isoflucypram in the gavage solution at the different concentrations tested is available (see document CA_B5 for further details).

Results

In the range-finding study, at 700 mg/kg bw/day, mean maternal body weight gain was decreased by 70% between GD6 and GD8; overall mean maternal body weight gain between GD6 and GD21 was marginally but not statistically significantly reduced by 6% compared to control. At necropsy, enlarged liver was noted in all treated dams at this dose level, and mean liver weight was increased by 87% compared to controls. At lower doses there were no effects on maternal body weight or body weight gain. Liver weight was stastically significantly increased, compared to controls, at 70 (by 22%) and 250 mg/kg/day (by 39%). No adverse changes were reported following limited external foetal examination at any dose.

Maternal toxicity

In the main study, at the top dose of 625 mg/kg bw/d, reduced food consumption (by 12% on GD 6-8; statistically significant) and body weight gain (BWG, by 43% on GD 6-8) were observed predominantly during GD 6-8. However, the reduction of BWG was not statistically significant.

Table B.6.6-15: Maternal food consumption, in g/day, during gestation after oral gavage administration of isoflucypram to rats

Gestation days		Isoflucypram, dose in mg/kg bw/day			
		0	**25**	**125**	**625**
1-6	g/day	26.1	25.7	26.7	25.3
	% control		98.5	102.6	96.9
6-8	g/day	26.4	26.1	26.6	23.1*
	% control		99.0	100.9	87.6
8-10	g/day	27.7	27.1	28.4	27.4
	% control		97.6	102.5	98.9
10-12	g/day	27.6	28.7	28.3	27.1
	% control		104.2	102.8	98.3
12-14	g/day	28.9	29.1	30.0	27.5
	% control		100.9	103.8	95.4
14-16	g/day	30.0	29.1	29.9	29.4
	% control		97.0	99.8	98.0
16-18	g/day	29.5	30.4	31.0	30.9
	% control		103.1	105.0	104.6
18-21	g/day	30.7	30.8	32.1	30.3
	% control		100.2	104.5	98.5

*: $p<0.05$,

Table B.6.6-16: Maternal body weight change during gestation after oral gavage administration of isoflucypram to rats

Gestation days		Isoflucypram, dose in mg/kg bw/day			
		0	**25**	**125**	**625**
0-6	g	43.9	44.4	42.1	41.2
	% control		101.1	95.9	93.8
6-8	g	4.6	4.7	5.4	2.6
	% control		102.2	117.4	56.5
8-10	g	11.6	8.7	13.2	12.9
	% control		75.0	113.8	111.2
10-14	g	23.8	27.1	22.8	21.4
	% control		113.9	95.8	89.9
14-18	g	45.7	47.1	46.2	44.9
	% control		103.1	101.1	98.2
18-21	g	58.1	55.4	58.4	55.0

Gestation days		Isoflucypram, dose in mg/kg bw/day			
		0	**25**	**125**	**625**
	% control		95.4	100.5	94.7
6-21	g	143.8	143.0	146.0	136.8
	% control		99.4	101.5	95.1

In the high dose dams, decreased bilirubin (by 84%, statistically significant) and ALP (by 34%, non- statistically significant), a higher incidence of enlarged livers (14/23 animals vs. 0/21 in the control group) and a higher absolute liver weight (by +44% compared to control) were observed. Histopathological examination found minimal to slight hepatocyte hypertrophy. All these effects are consistent with the findings observed in repeated dose toxicity dietary studies at lower doses of isoflucypram.

Table B.6.6-17: Mean maternal carcass weight, liver weight, and thyroid weight after oral gavage administration of isoflucypram to rats

		Isoflucypram, dose in mg/kg bw/day			
		0	**25**	**125**	**625**
Maternal carcass weight	g	348.5	344.6	348.9	341.3
	% control		98.9	100.1	97.9
Liver wt	g	14.1	14.0	15.5	20.3
	% control		99.1	109.8	143.8
Macroscopy: N examined		21	21	23	23
Liver, enlarged		0	0	0	14
Microscopy: N examined		10	0	10	10
Hepatocellular hypertrophy, centrilobular, diffuse					
Minimal		0		0	5
Slight		0		0	5
Total		0		0	10
Thyroid wt	g	0.0166	0.0154	0.0164	0.0170
	% control		92.8	98.5	102.2
Microscopy: N examined		10	0	10	10
Follicular cell hypertrophy, diffuse					
Minimal		0		0	1
Total		0		0	1

Bilirubin was also decreased at the mid-dose. As explained in the short-term toxicity section, the decrease in bilirubin and ALP, although treatment-related, is not considered to be adverse as this decrease was the consequence of liver UDPGT enzyme induction by the test substance. There were no effects on body weight gain, food consumption, organ weights, macroscopic or microscopic findings at the mid and low doses. Overall, maternal toxicity (decreased BWG and food consumption and increased liver weight with associated hypertrophy) was seen at the top dose of 625 mg/kg bw/d.

In the kinetic investigations in maternal animals, the plasma concentration of isoflucypram was only marginally above the limit of quantification at all doses. However, there was a dose-related increase in the concentrations of two metabolites (BCS-CX99798 or M58 and BCS-CX99799 or M11), indicating that isoflucypram had been absorbed and metabolized after administration (see ADME section – B.6.1.).

Table B.6.6-18: Mean concentrations of isoflucypram and the metabolites BCS-CX99798 (M58) and BCS-CX99799 (M11) in maternal plasma at necropsy

	Isoflucypram, dose in mg/kg bw/day			
	0	**25**	**125**	**625**
Isoflucypram	< LOQ	< 0.012	0.015 ± .007	0.017 ± 0.003
BCS-CX99798 (M58)	< LOQ	0.039 ± 0.020	0.147 ± 0.037	0.676 ± 0.260
BCS-CX99799 (M11)	< LOQ	0.339 ± 0.197	0.273 ± 0.106	0.607 ± 0.306

Developmental toxicity

No treatment-related effects on the number of live foetuses, number of implantation sites per dam, post-implantation losses, early and late resorptions, foetal deaths or sex distribution of foetuses were observed at any

dose. Foetal body weight was slightly decreased at 625 mg/kg bw/d; this decrease was only statistically significant for female fetuses.

Table B.6.6-19: Caesarean section data from dams administered isoflucypram by oral gavage during gestation

Maternal data	Isoflucypram, dose in mg/kg bw/day			
	0	**25**	**125**	**625**
No. Animals assigned	23	23	23	23
No. animals pregnant	21	22	21	21
Pregnancy rate, %	91	96	100	100
No. Animals non-pregnant	2	1	0	0
Maternal wastage:				
Intercurrent death or sacrifice, total	0	1	0	0
Intercurrent death or sacrifice, pregnant	0	1	0	0
Premature delivery	0	0	0	0
Intercurrent death or sacrifice, non-pregnant	0	0	0	0
Uterine data at scheduled sacrifice				
Total no. corpora lutea	382	367	410	415
Corpora lutea per dam	18.2	17.5	17.8	18.0
Total no. implantations	328	320	352	369
Implantations per dam	15.6	15.2	15.3	16.0
Total no. litters	21	21	23	23
Total no. live foetuses	307	306	333	343
Live fetuses per dam	14.6	14.6	14.5	14.9
Total no. dead fetuses	0	0	0	0
Total no. early resorptions	21	13	15	25
Early resorptions per dam	1.0	0.6	0.7	1.1
Total no. late resorptions	0	1	4	1
Late resorptions per dam	0	0.04	0.2	0.05
Litters with total resorptions	0	0	0	0
Mean fetal wt, g, combined sexes	5.59	5.63	5.63	5.37
Mean fetal wt, g, males	5.70	5.75	5.80	5.51
Mean fetal wt, g, females	5.47	5.48	5.49	5.21*
Sex ratio, % males	51.1	56.9	46.8	53.4
Sex ratio, % males per litter	51.0	56.3	47.4	53.9
Pre-implantation loss per dam, %	13.33	12.31	13.81	10.75
Post-implantation loss per dam, %	6.54	4.32	5.25	7.10

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

No treatment-related malformations (external, skeletal or visceral), external variations or retardations were observed at any dose level. Slight increases in some common skeletal and visceral variations were seen at the top dose of 625 mg/kg bw/d. Foetal skeletal variations included delayed ossification in the zygomatic arch (statistically significant and above appropriate HCD), squamosal (non-statistically significant but above appropriate HCD), hyoid centrum (statistically significant and above appropriate HCD), femur (statistically significant but within appropriate litter incidence HCD) and humerus (non-statistically significant but above appropriate HCD). Foetal visceral variations included distended bladder (non-statistically significant but above appropriate HCD), dilated renal pelvis (unilateral/bilateral, statistically significant and above appropriate HCD) and thymic remnant (unilateral/bilateral, statistically significant but within appropriate HCD). All of these variations occurred in the presence of signs of maternal toxicity and at this dose are considered to be treatment-related but of minimal toxicological significance and the unspecifc, secondary consequence of the maternal toxicity observed at the top dose of 625 mg/kg bw/d.

Some of these common variations were also slightly increased (non statistically significantly) at the mid dose of 125 mg/kg bw/d. However, at this dose, with the exception of the delayed ossification of the squamosal, the increases were within the appropriate HCD. On this basis, the increased incidences of these variations at the mid dose are considered to represent normal variation unrelated to treatment with isoflucypram. As for the delayed ossification of the squamosal, it is noted that the foetal incidence was within the HCD. The RMS concludes that

in isolation, the slight increase in the litter incidence of the delayed ossification of the squamosal above HCD at 125 mg/kg bw/d is of no toxicological significance.

Overall, there were treatment-related increased incidences of some skeletal and visceral variations at the top dose. These increases were the unspecifc, secondary consequence of the maternal toxicity observed at the top dose of 625 mg/kg bw/d.

Table B.6.6-20: Incidences of selected foetal visceral and skeletal variations in rats

Dose (mg/kg bw/d)	0	25	125	625	Appropriate lab HCD[1] Foetal incidence (%)	Appropriate lab HCD[1] Litter incidence (%)
Observations	No.of foetuses examined/ litter number					
	148/21	148/21	159/23	167/23		
	% foetuses affected / % litters affected					
Visceral variations						
Thymic remnant present (uni/bi)	2 / 14.3	4.1 / 19	4.4/ 26.1	7.2* / 34.8	0 – 10.3 mean = 4.5	8.7- 40.9 mean = 22.4
Bladder, distended	0 / 0	0 / 0	0 / 0	1.8 / 4.3	0 - 0	0 – 0
Dilated renal pelvis (uni/bi) (1< severe)	0 / 0	2 / 4.8	1.3 / 8.7	4.2* / 26.1*	0 – 3.2 mean = 1.4	0 – 21.7 mean = 8.8
Skeletal variations- incomplete ossification						
At least one bone of zygomatic arch (uni/bi)	2.5 / 14.3	3.8 / 14.3	5.7 / 30.4	8.5* / 52.2*	0 – 8.8 mean = 2	0 – 34.8 mean = 12.5
Squamosal (uni/bi)	0.6 / 4.8	1.3 / 4.8	1.7 / 13.0	2.3 / 17.4	0 – 2 mean = 0.6	0 – 9.1 mean = 4
Hyoid centrum	2.5 / 19.0	1.3 / 9.5	6.3/ 34.8	8.0* / 39.1	0 – 7.9 mean = 2.7	0 – 34.8 mean = 19.1
Femur (uni/bi)	1.3 / 9.5	2.5 / 19.0	4.6 / 21.7	8.5 **/ 21.7	0.6 – 6.4 mean = 2.5	4.3 – 26.1 mean = 12.7
Humerus (uni/bi)	0 / 0	0 / 0	2.3 / 13.0	1.7 / 8.7	0 – 1.3 mean = 0.5	0 – 4.5 mean = 2.2

*/**Statistically significant;

[1]Lab HCD data from 19 gavage developmental toxicity studies conducted in SD rats between 2006 and 2017.

Conclusion

In this standard OECD 414 guideline compliant rat developmental study no malformations (external, skeletal or visceral) were observed at any dose. Foetal weight was reduced in females at the top dose of 625 mg/kg bw/d. Some statistically significant increases in skeletal and visceral variations were also observed at the top dose only. These common variations are considered to represent a slight developmental delay (related to the decreased foetal body weight) of minimal toxicological significance and the unspecifc, secondary consequence of the maternal toxicity (reduced BWG and food consumption and increased liver weight with associated hypertrophy) observed at the top dose of 625 mg/kg bw/d. No maternal toxicity was observbed at lower dose levels. Overall, it can be concluded that isoflucypram is not a specific developmental toxicant in rats. Based on these findings, the NOAELs proposed by the RMS for developmental and maternal toxicity in the rat are 125 mg/kg bw/d, based on the lack of relevant effects at these dose levels.

([redacted] *2017b)*

Rabbits

Study	Developmental Toxicity
Reference	[redacted] (2017)

Test facility	[redacted]
Report reference	Study number Sponsor reference SA 15122
Guideline(s)	OECD 414
Deviations from the guideline	None
GLP	Yes. Signed QA and GLP certificates provided
Test material	Isoflucypram technical Batch: 2013-006492, Purity 94.2% w/w,
Study acceptable	Yes

Methods

Isoflucypram was administered to groups of female rabbits (New Zealand White strain, 23/dose) by oral gavage on days 6-28 of gestation at doses of 0, 10, 70, 500 mg/kg bw/d, in aqueous 0.5% methylcellulose 400. The test substance was stable in the chosen vehicle. A fully validated method of analysis for isoflucypram in the gavage solution at the different concentrations tested is available (see document CA_B5 for further details).

The doses employed were established from a range-finding study (New Zealand White strain) in which isoflucypram was administered to pregnant rabbits at doses of 0, 50, 150, and 450 mg/kg bw/d on days 6 to 28 of gestation. Mean food consumption was statistically significantly decreased on gestation days 8-10, while decreases from gestation days 6-8 and gestation days 10-12 were not statistically significant. At the beginning of the treatment period (gestation days 6-8), there was a mean maternal body weight loss of 30g, compared to a gain of 30g in the control group. Otherwise, body weight parameters including corrected body weight change were unaffected by the treatment.There were no adverse effects on maternal mortality, any litter parameters, or fetal body weight. Liver weight was increased by approximately 20-21% at all dose levels. There were no treatment-related findings at fetal external examination.

Results

Maternal toxicity

In the main study, no mortality (dams) and no treatment-related clinical signs were observed at any dose level. At 500 mg/kg bw/d, two dams aborted prior to the end of the study; this was considered to be the consequence of the severe maternal toxicity (body weight loss) observed at this dose (see below). In the high dose group, reduced food consumption (by 14% on GD 10-14, statistically significant) and body weight loss (by 68% on GD 6-8, statistically significant) were observed during the initial part of the dosing period. In addition, body weight gain was reduced overall by 27% during GD 6-29 (non-statistcially significant). In the 70 mg/kg bw/d dose group mean overall body weight gain was also lower (by 12% on GD 6-29) compared to the control group; however, this difference was not statistically significant. In addition, in isolation and given its magnitude, it is not considered to be adverse by the RMS.

Table B.6.6-21: Mean maternal food consumption in rabbits administered isoflucypram by oral gavage during gestation

Gestation day		Isoflucypram, dose in mg/kg bw/day			
		0	10	70	500
6-8	g/day	149.3	168.1	160.3	127.2
	% control		112.6	107.4	85.2
8-10	g/day	162.7	170.2	159.7	126.4
	% control		104.6	98.1	77.7
10-14	g/day	143.7	150.6	141.0	123.8*
	% control		104.8	98.1	86.1
14-18	g/day	129.2	145.1	135.1	120.5
	% control		112.3	104.6	93.3
18-22	g/day	145.9	149.2	136.4	135.8
	% control		102.3	93.5	93.1
22-26	g/day	99.7	114.2	110.0	103.2
	% control		114.6	110.4	103.6
26-29	g/day	99.3	108.6	100.8	100.9
	% control		109.4	101.5	101.6

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Table B.6.6-22: Mean maternal body weight change in rabbits administered isoflucypram by oral gavage during gestation

Gestation day		Isoflucypram, dose in mg/kg bw/day			
		0	10	70	500
6-8	kg	0.028	0.032	0.007	-0.019**
	% control		114.3	25.0	-67.9
8-10	kg	0.051	0.035	0.031	0.024
	% control		68.6	60.8	47.1
10-14	kg	0.076	0.082	0.073	0.057
	% control		107.9	96.1	75.0
14-18	kg	0.058	0.049	0.044	0.044
	% control		84.5	75.9	75.9
18-22	kg	0.044	0.065	0.047	0.048
	% control		147.7	106.8	109.1
22-26	kg	0.043	0.057	0.064	0.036
	% control		132.6	148.8	83.7
26-29	kg	0.074	0.070	0.063	0.085
	% control		94.6	85.1	114.9
6-29	kg	0.375	0.390	0.330	0.275
	% control		104.0	88.0	73.3

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

The high dose animals showed a higher absolute liver weight (+16%). There were no adverse effects in maternal animals at 10 and 70 mg/kg bw/d.

Table B.6.6-23: Mean maternal carcass weight and liver weight after oral gavage administration of isoflucypram to rabbits

		Isoflucypram, dose in mg/kg bw/day			
		0	10	70	500
Maternal carcass weight	g	3306.3	3344.4	3287.0	3291.5
	% control		101.2	99.4	99.6
Liver wt	g	94.5	93.7	101.4	109.4**
	% control		99.2	107.3	115.8

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Developmental toxicity

At caesarean section, mean number of early and late resorptions, percentage of post-implantation loss, mean litter size, mean foetal body weight and sex ratio were comparable between trated and control groups. No dead foetuses were observed. There were no treatment-related external, visceral or skeletal malformations, variations or retardations.

Table B.6.6-24: Caesarean section data from does administered isoflucypram by oral gavage during gestation

Maternal data	Isoflucypram, mg/kg bw/day			
	0	10	70	500
No. Animals assigned	23	23	23	23
No. animals pregnant	23	22	23	23
Pregnancy rate, %	100	96	100	100
No. Animals non-pregnant	0	1	0	0
Maternal wastage:				
Intercurrent death or sacrifice, total	0	0	0	2
Intercurrent death or sacrifice, pregnant	0	0	0	2
Premature delivery	0	0	0	0
Intercurrent death or sacrifice, non-pregnant	0	0	0	0
Abortions	0	0	0	2

Maternal data	Isoflucypram, mg/kg bw/day			
	0	**10**	**70**	**500**
Uterine data at scheduled sacrifice				
Total no. corpora lutea	279	248	276	240
Corpora lutea per dam	12.1	11.3	12.0	11.4
Total no. implantations	243	199	226	204
Implantations per dam	10.6	9.0	9.8	9.7
Total no. litters	23	22	23	21
Total no. live fetuses	226	189	211	186
Live fetuses per dam	9.8	8.6	9.2	8.9
Total no. dead fetuses	0	0	0	0
Total no. early resorptions	10	6	8	6
Early resorptions per dam	0.4	0.3	0.3	0.3
Total no. late resorptions	7	4	7	12
Late resorptions per dam	0.3	0.2	0.3	0.6
Litters with total resorptions	0	0	0	0
Mean fetal wt, g, combined sexes	39.7	41.8	39.9	39.7
Mean fetal wt, g, males	40.0	42.6	41.5	39.1
Mean fetal wt, g, females	39.5	41.1	37.7	39.9
Sex ratio, % males	45.6	47.1	49.3	44.1
Sex ratio, % males per litter	45.1	45.8	51.0	44.4
Pre-implantation loss per dam, %	12.2	18.9	15.7	14.3
Post-implantation loss per dam, %	6.1	4.7	8.6	8.8

Table B.6.6-25: Incidence of selected external fetal variations in rabbits

		Isoflucypram, dose in mg/kg bw/day			
		0	**10**	**70**	**500**
N examined	Litters	23	22	23	21
	Fetuses	226	189	211	186
Abdomen distended	Litters	1	0	0	0
	%	4.3	0.0	0.0	0.0
	Fetuses	1	0	0	0
	%	0.4	0.0	0.0	0.0
Forepaws (uni/bi): malrotated: outward	Litters	0	1	1	2
	%	0.0	4.5	4.3	9.5
	Fetuses	0	1	1	2
	%	0.0	0.5	0.5	1.1
Tail: short	Litters	0	0	0	1
	%	0.0	0.0	0.0	4.8
	Fetuses	0	0	0	1
	%	0.0	0.0	0.0	0.5
Head: subcutaneous hemorrhage	Litters	0	0	0	1
	%	0.0	0.0	0.0	4.8
	Fetuses	0	0	0	1
	%	0.0	0.0	0.0	0.5

Table B.6.6-26: Incidence of selected fetal skeletal variations in rabbits

		Isoflucypram, dose in mg/kg bw/day			
		0	**10**	**70**	**500**
N examined	Litters	23	22	23	21
	Fetuses	226	189	211	186
	Heads	118	99	110	97
Nasal (uni/bi) / frontal (uni) / parietal (uni/bi): split	Litters	2	4	3	3
	%	8.7	18.2	13.0	14.3
	Fetuses	2	4	4	3
	%	1.69	4.04	3.64	3.09
Anterior and / or posterior fontanelles: enlarged	Litters	1	1	3	2
	%	4.35	4.55	13.0	9.52
	Fetuses	1	2	3	2
	%	0.85	2.02	2.73	2.06
Hyoid centrum: incomplete ossification or unossified	Litters	6	8	12	6
	%	26.1	36.4	52.2	28.6
	Fetuses	10	12	19	9
	%	8.47	12.1	17.3*	9328
Extra ossification point (uni) and / or cervical rib (uni/bi): short on 7th cervical vertebra	Litters	3	4	1	4
	%	13.0	18.2	4.35	19.0
	Fetuses	3	4	2	4
	%	1.33	2.12	0.95	2.15
Extra sternebral ossification site	Litters	0	0	2	1
	%	0.0	0.0	8.7	4.76
	Fetuses	0	0	2	3
	%	0.0	0.0	0.95	1.61
5th and / or 6th sternebrae: unossified	Litters	10	13	11	12
	%	43.5	59.1	47.8	57.1
	Fetuses	21	33	31	24
	%	9.29	17.5*	14.7	12.9
1st metacarpal(s): incomplete ossification or unossified	Litters	12	5	6	11
	%	52.2	22.7	26.1	52.4
	Fetuses	19	13	21	22
	%	8.41	6.88	9.95	11.8

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Conclusion

Overall, from the results of this study there is no evidence that isoflucypram is a developmental toxicant in rabbits up to the top dose of 500 mg/kg bw/d at which severe maternal toxicity (2 abortions, initial body weight loss and initial reduction in food consumption, overall decrease in body weight gain during the dosing period and increased liver weight) occurred. Based on these data, the NOAELs proposed by the RMS for developmental and maternal toxicity are 500 and 70 mg/kg bw/d, respectively based on the lack of relevant effects at these dose levels.

(*2017)*

B.6.6.3. Summary of reproductive toxicity

The reproductive toxicity of isoflucypram has been investigated in a standard guideline dietary 2-generation study in rats and in guideline gavage pre-natal developmental toxicity studies, one in rats and one in rabbits.

Effects on Sexual Function and Fertility

The potential of isoflucypram to adversely affect sexual function and fertility has been well investigated in a standard 2-generation dietary study, conducted in rats.

There were no effects of treatment on mating behaviour, fertility, litter size, oestrus cycle, ovarian primordial follicle counts and gestation up to the top dose of 1200/600 ppm (93 – 104 mg/kg bw/d) at which parental and offspring toxicity occurred. In addition, examination of the reproductive organs did not reveal any treatment-related changes. Specific investigations of the spermatogenic cycle did not find any cell or stage-specific abnormalities at any dose level. Therefore a **NOAEL for reproductive toxicity** of **93-104 mg/kg bw/d** (highest dose tested) can be identified from this study.

In relation to general toxicity in parental animals and offspring, liver and thyroid weights (with no associated histopathology) were adversely increased from the mid dose of 450/225 ppm (equivalent to 34.1-40.8 mg/kg/day) in adults and pups in both generations. Clinical-chemistry parameters associated with liver toxicity were also affected from the mid dose in parental animals of both generations. In addition, food consumption was decreased in F1 adult females at the top dose. There were no adverse effects in parental animals and offspring at the lowest dose tested of 150/75 ppm (11-14 and mg/kg/d). Therefore a **NOAEL** of **11-14 mg/kg bw/d** can be identified for **parental and offspring toxicity** from this study.

Overall, classification of isoflucypram for effects on fertility is not warranted (see also aligned CLH report).

Developmental toxicity

The developmental toxicity of isoflucypram has been investivated in guideline gavage pre-natal developmental toxicity studies, conducted in rats and rabbits. Additional information on the developmental toxicity potential of isoflucypram is also available from the rat 2-generation study.

In the rat developmental study, no malformations (skeletal or visceral) were observed at any dose. Foetal weight was reduced in females at the top dose of 625 mg/kg bw/d. Some skeletal and visceral variations were also observed at the top dose only. These common variations are considered to be of minimal toxicological significance and the unspecifc, secondary consequence of the maternal toxicity (reduced BWG and food consumption and increased liver weight with associated hypertrophy) observed at the top dose of 625 mg/kg bw/d. No maternal toxicity was observed at lower dose levels. Overall, it can be concluded that isoflucypram is not a specific developmental toxicant in rats. Based on these findings, the **NOAELs** proposed by the RMS for **developmental and maternal toxicity** in the rat are **125 mg/kg bw/d**, based on the lack of relevant effects at these dose levels.

In the rabbit developmental study, there was no evidence that isoflucypram was a developmental toxicant up to the top dose of 500 mg/kg bw/d at which severe maternal toxicity (2 abortions, initial body weight loss and initial reduction in food consumption, overall decrease in body weight gain during the dosing period and increased liver weight) occurred. Based on these data, the **NOAEL**s proposed by the RMS for **developmental and maternal toxicity** in rabbits are **500 and 70 mg/kg bw/d**, respectively based on the lack of relevant effects at these dose levels.

In addition, in the rat 2-generation study, there were no effects of treatment on pup survival, sex ratio, pup bodyweight, preputial separation or ano-genital distance (AGD) up to the top dose of 1200/600 ppm (93 – 104 mg/kg bw/d) at which parental and offspring toxicity occurred.

The mean age at vaginal opening for F1 females in the 1200/600 ppm group occurred five days later than in controls. The body weight of these F1 females was also significantly increased. In the presence of an increase in body weight (usually delayed vaginal opening is associated with reduced body weight), it is difficult to establish the toxicological significance of the observed delay in vaginal opening. However, in the absence of any effects on other developmental landmarks and on AGD and considering that these females went on to mate successfully and produce the F2 generation, the RMS concludes that it is most likely this observation was a chance finding. In addition, no effects on vaginal opening were seen up to the high gavage dose of 400 mg/kg bw/d for 20 days in a modified rat uterotrophic assay in immature animals ([redacted] 2011; see section B.6.8.3) which included specific investigations of vaginal opening.

Overall, classification of isoflucypram for developmental toxicity is not warranted (see also aligned CLH report).

The table below summarises the relevant NOAEL and LOAEL values identified in the reproductive toxicity studies.

Table B.6.6-27: Summary of reproductive toxicity studies with isoflucypram

Study, guideline and reference	**Species and dose tested**	**NOAEL mg/kg bw/d**	**LOAEL mg/kg bw/d**	**Adverse effects at the LOAEL**
2-gen study (dietary) OECD 416 ([redacted] 2018)	Rat (Wistar) 0, 150/75, 450/225, 1200/600 ppm Equivalent to 0, 11-14, 34-44, 93-140 mg/kg bw/d	*Reproductive* 93-140 (1200/600 ppm) *Parental* **11-14 (150/75 ppm)** *Offspring* 11-14 (150/75 ppm)	*Reproductive* >93-140 (>1200/600 ppm) *Parental* 34-44 (450/225 ppm) *Offspring* 34-44 (450/225 ppm)	*Reproductive* No adverse effects up to the top dose *Parental* ↑ liver and thyroid wt; effects on some clinical-chemistry parameters indicative of liver toxicity *Offspring* ↑ liver wt
Pre-natal dev tox study (gavage) OECD 414 ([redacted] 2017b)	Rat (SD) 0, 25, 125, 625 mg/kg bw/d	*Developmental* 125 *Maternal* 125	*Developmental* 625 *Maternal* 625	*Developmental* ↓ foetal wt, ↑ incidence of skeletal and visceral common variations *Maternal* ↓ bwg, ↓ food consumption, ↑ liver wt with associated hypertrophy
Pre-natal dev tox study (gavage) OECD 414 ([redacted] 2017)	Rabbit (NZW) 0, 10, 70, 500 mg/kg bw/d	*Developmental* 500 *Maternal* 70	*Developmental* >500 *Maternal* 500	*Developmental* No adverse effects up to top dose *Maternal* 2 abortion, initial body weight loss, ↓ overall bwg, ↓ initial food consumption, ↑ liver wt

Overall classification of isoflucypram for reproductive toxicity is not warranted. Reproductive toxicity has been adequately investigated in studies that comply with OECD guidelines 414 (2001) and 416 (2001).

B.6.7. NEUROTOXICITY

A guideline acute neurotoxicity study in the rat is available.

B.6.7.1. Neurotoxicity studies in rodents

Study	Isoflucypram - An acute neurotoxicity study in the rat by oral administration
Reference	[redacted] 2017
Date performed	11 February 2015 – 20 March 2015
Test facility	[redacted]
Report reference	SA 15004

Guideline(s)	OECD guideline 424
Deviations from the guideline	None
GLP	Yes. Signed QA and GLP certificates provided
Test material	isoflucypram technical. Batch: 2013-006492, Purity 94.2% w/w, CAS No 1255734-28-1.
Study acceptable	Yes

Methods

Isoflucypram was administered once by oral gavage to groups of Wistar rats (12 per sex per group) at dose levels of 0, 200, 600, and 2000 mg/kg bw/day in 0.5% aqueous methylcellulose 400. Neurotoxicity assessment including a functional observational battery (FOB) and spontaneous motor activity was performed on four occasions (during pre-study phase, approximately 6 hours after dosing, and then 7 and 14 days after dosing). All surviving animals were subjected to a complete necropsy. At least 6 animals per sex per group were subjected to neuropathological investigation with selected organs weighed and a range of organs fixed and examined microscopically.

Results

There was no mortality or any treatment-related clinical signs at any dose tested.

There were no effects on either body weight or body weight gain in either males or females at any dose level compared to controls. Although there was a slight tendency towards an increase in body weight gain among treated groups, this is attributed to a high heterogeneity of individual values, especially in control groups, leading to lower mean values in controls. Additionally, there was no statistical significance of the increase and no relationship to dose, thus the slight changes observed in body weight were considered to be not relevant and not related to dose. Nor was there an effect on mean brain weight in any treated animal compared to controls.

Table B.6.7-1: Mean body weight and body weight change in males and females administered a single dose of isoflucypram

		Isoflucypram, dose in mg/kg bw							
		Males				Females			
		0	200	600	2000	0	200	600	2000
Body wt, g	1	325	326	323	319	224	218	224	225
	7	354	357	354	354	233	235	236	240
	14	378	391	391	383	248	251	248	251
Body wt gain, g	1-7	29	31	31	35	10	18	13	14
	1-14	53	65	68	64	24	33	25	25

No treatment-related effects were observed in the FOB at any dose level in either males or females on home cage observations, observations during handling, open-field behavior, or sensory reactivity tests.

In the neurotoxicology assessments, incidental changes in mean landing foot splay and grip strength were not considered treatment related as there was no consistency across either time period or dose group. Furthermore, statistically significant increases or decreases in measurements were observed during the pre-study examinations. There were no relevant changes in rectal temperature, body weight or body weight gain or in the neuropathological examination in any treated group compared to controls.

Table B.6.7-2: Landing foot splay, and fore- and hindlimb grip strength in male and female rats administered isoflucypram by oral gavage

		Isoflucypram, dose in mg/kg bw							
		Males				Females			
		0	200	600	2000	0	200	600	2000
Landing foot splay, cm	Pre-study	9.05	7.12*	6.88*	7.29	6.56	7.62	7.29	7.30
	1	10.06	8.68	9.59	9.38	7.74	7.81	8.33	8.71
	7	10.59	8.46*	9.02	10.29	6.90	7.68	7.91	8.14
	14	10.37	8.88	9.11	8.91	7.54	7.98	8.72	9.34*
Forelimb grip strength, g	Pre-study	526.8	479.4	529.0	500.6	494.4	547.8	555.8	498.8
	1	667.1	592.3	592.2	573.7	561.4	553.0	625.5	598.7
	7	666.1	500.5**	555.0	590.8	481.9	525.4	545.3	527.1
	14	574.8	549.9	594.3	583.2	427.9	463.1	533.5	432.9
Hindlimb grip strength, g	Pre-study	416.5	421.3	398.8	378.1	430.3	451.3	490.8	438.3
	1	485.3	502.0	472.0	449.8	429.2	454.3	481.1	462.8

		Isoflucypram, dose in mg/kg bw							
		Males				Females			
		0	200	600	2000	0	200	600	2000
	7	534.8	514.3	483.8	469.6	395.4	447.3	449.9	430.3
	14	605.5	618.1	541.1	483.8**	412.8	414.7	449.4	409.4

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

No relevant changes were recorded in overall motor activity in any test groups compared to the control group at any time point. In addition, the general pattern of motor activity within each test session was similar between test groups and the control group, showing no indication of a treatment-related effect.

There were no macroscopic findings in any dose group in either males or females, either in animals of the neuropathology group or in other animals, nor were any treatment-related microscopic findings noted in either males or females at any dose level.

Conclusion

Overall, in a guideline oral study, isoflucpyram did not show any potential for acute neurotoxicity in rats up to the limit dose of 2000 mg/kg bw. No generalised toxicity was evident up to the top dose. Thus, a NOAEL of 2000 mg/kg was identified for acute neurotoxicity and generalised toxicity from this study.

([redacted] *2017)*

B.6.7.2. Delayed polyneuropathy studies

No studies are available and none are required.

B.6.7.3. Summary of neurotoxicity

The neurotoxicity potential of isoflucypram has been investigated in a guideline oral acute neurotoxicity study in the rat. In this study, isoflucpyram did not show any potential for neurotoxicity up to the limit dose of 2000 mg/kg bw.

B.6.8. OTHER TOXICOLOGICAL STUDIES

B.6.8.1. Toxicity studies on metabolites and relevant impurities

B.6.8.1.1. Toxicity studies on metabolites

The following metabolites were selected for potential inclusion in the residue definitions based on their significant occurrence in the plant and livestock metabolism studies:

- Isoflucypram-propanol (M01) and its conjugates (M18, M19 & M21)
- Isoflucypram-2-propanol (M02) and its conjugates (M20 & M22)
- Isoflucypram-desmethyl-propanol (M06) and its conjugates (M37 & M41)
- Isoflucypram-desmethyl-1,2-propandiol (M07) and its conjugate (M36)
- Isoflucypram-desmethyl-carboxylic acid (M11)
- Isoflucypram-carboxylic acid (M12)

To assess the toxicological properties of these metabolites, all the available data (including data relating to isoflucypram) were considered. These included presence of these metabolites in rat ADME and toxicity studies performed with the parent, structural similarity to the parent and in silico genotoxicity assessment. No specific toxicity studies are available on these metabolites.

Presence of selected metabolites in rat ADME and toxicity studies

Review of the ADME studies conducted with isoflucypram has revealed that most of the above metabolites have been detected in rats. It is generally accepted that the toxicity of a metabolite is covered by the toxicity data of the parent if that individual metabolite contributes to ≥ 10% of the administered dose found in excreta, plasma and tissues in ADME studies. None of these metabolites were detected in urine at levels greater than 10% of the administered dose (as an individual metabolite); however, the majority were detected in bile in the rat ADME

studies at levels ranging from 2% to 16% of the administered dose and all (except M07 – not investigated) were seen in plasma in the 2-year rat study, at similar or greater amounts than the parent. M11 was also observed in plasma at levels similar to or greater than the parent in the repeated-dose rat, mouse and dog studies, the 2-generation study in rats and in the rat and rabbit developmental toxicity studies. Presence of these metabolites in bile is considered evidence of systemic availability as, for isoflucypram, the most critical target organ of toxicity is the liver.

Metabolite **M01** was found in the bile of rats (ADME study) at a level of 10.25% in males and 15.73% in females. Furthermore, M01 was detected in the 2-year rat study in plasma at similar levels to the parent. Therefore, the toxicity of metabolite M01 can be considered covered by the toxicity data of the parent. As the metabolites M18, M19 and M21 (conjugates of M01) are cleaved into M01 in the stomach after oral ingestion, then the toxicity of these metabolites is also be covered by the toxicity of M01 and hence by the toxicity data of the parent.

Metabolite **M11** was detected in plasma at similar or higher concentrations than the parent in the repeated-dose rat, mouse and dog studies, the 2-generation rat study, the rat and rabbit developmental toxicity studies and the rat 2-year study. Therefore, the toxicity of this metabolite is also covered by the toxicity data of the parent.

Metabolites **M02**, **M06** and **M12** (and their conjugates) were also found in rat plasma, taken from the 2-year study, at levels that were similar to or greater than those of the parent. Therefore, it is acceptable to use the toxicity data from the parent compound for these metabolites.

Metabolite **M07** was not investigated in rat plasma from the 2-year study. However, it is noted that M07 is the hydroxylated form of M06.

Overall, metabolites M01, M02, M06, M11 and M12 were detected in rat plasma at levels similar or greater than the parent. Therefore, the toxicity of these metabolites is covered by the toxicity data of the parent.

Structural similarity of the selected metabolites with the parent

Metabolites M01, M02, M06, M07, M11, M12 are structurally similar to one another and do not differ significantly from the parent. The metabolites M01 and M02 differ from the parent only by one hydroxylation; the metabolite M06 corresponds to the demethylated form of M01; the metabolite M07 contains one more hydroxy compared with M06; and the metabolite M12 corresponds to the demethylated form of M11.

These slight variations, comprising demethylation, hydroxylation and/or oxidation would not be expected to trigger significantly different toxicity profiles when compared with the parent. Therefore, the metabolites M01, M02, M06, M07, M11 and M12 (and their conjugates) can be considered covered by the toxicity data of the parent compound based on structural similarities.

***In silico* genotoxicity assessment of selected metabolites**

The applicant has subjected the selected metabolites and the active compound (isoflucypram) to *in silico* assessment for genotoxicity using the following suite of tools:

- Derek Nexus, using Nexus version 2.2.0, Derek Nexus version 6.0.0, and Knowledge Base 2018 1.1; all alerts were run;
- Leadscope Model Applier 2.2, using the Genetox Suite;
- Toxtree version 2.6.13, using only the models for Cramer class, Ames mutagenicity, and in vivo mouse micronucleus;
- TopKat, running within a server installation of BioVia's Discovery Studio, version 17.2.0, using only the alert for Ames mutagenicity;
- Vega version 1.1.4, using only the CAESAR (version 2.1.13), SarPy/IRFMN (version 1.0.7), ISS (version 1.0.2), and KNN/Read-across (version 1.0.0) models

The findings are summarised below.

Table B.6.8-1: QSAR genotoxicity predictions for isoflucypram and its selected metabolites

Software	Endpoint/model		Isoflucypram	M01[2]	M02[3]	M06[4]	M07[5]	M11	M12
Derek	Genotoxicity alerts		Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
Leadscope	Clast. *In vitro*	Chrom Ab CHL v. 2	Neg	Neg	Pos	Neg	Pos	Pos	Pos
		Chrom Ab CHO v. 2	Neg	Neg	Neg	Neg	Neg	Neg	Neg
		SCE CHO v. 1	Neg	Neg	Neg	Neg	Neg	Neg	Neg
		SCE Comp v. 1	Neg	Neg	Neg	Neg	Neg	Neg	Neg
		SCE Other v. 1	Pos	Pos	Pos	Pos	Pos	Neg	Neg
	Clast. *In vivo*	Chrom Ab Comp v. 1	Neg	Neg	Neg	Neg	Neg	Neg	Neg
		Chrom Ab Other v. 1	Neg	Neg	Neg	Neg	Neg	Neg	Neg
		Chrom Ab rat v. 1	Neg	Neg	Neg	Neg	Neg	Neg	Neg
		Mouse micronuc v. 2	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	Gene mutation	HPRT Mut v. 1	Neg	Neg	Neg	Neg	Neg	Neg	Neg
		Mouse lymphoma Act v. 2	Neg	Neg	Neg	Neg	Neg	Neg	Neg
		Mouse lymphoma unact v. 2	Neg	Neg	Neg	Neg	Neg	Neg	Neg
		Rodent DL mut v. 1	Neg	Neg	Neg	Neg	Neg	Neg	Neg
		Rodent mut v. 1	Neg	Neg	Neg	Neg	Neg	Neg	Neg
		E coli – Sal 102 A-T Mut v. 1	Neg	Neg	Neg	Neg	Neg	Neg	Neg
		Salmonella mut v. 3	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Toxtree	Cramer class		III	III	III	III	III	III	III
	Ames mutagenicity		No alerts	No alerts	No alerts	No alerts	No alerts	No alerts	No alerts
	In vivo mouse micronucleus		H-acceptor	H-acceptor	H-acceptor	H-acceptor	H-acceptor	H-acceptor	H-receptor
TopKat	Ames mutagenicity		Neg	Neg	Neg	Neg	Neg	Neg	Neg

[2] And its conjugates M18, M19 & M21
[3] And its conjugates M20 & M22
[4] And its conjugates M37 & M41
[5] And its conjugate M36

Software	Endpoint/model	Isoflucypram	M01[2]	M02[3]	M06[4]	M07[5]	M11	M12
Vega	Ames mutagenicity, CAESAR model	Neg	Pos for M01 (neg for all conjugates)	Pos for M02 (neg for its conjugates)	Neg	Neg	Neg	Pos
	Ames mutagenicity, SarPy/IRFMN model	Pos	Pos	Pos	Pos	Pos	Pos	Pos
	Ames mutagenicity, ISS model	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	Ames mutagenicity, KNN/Read-across model	Neg	Neg	Neg	Neg	Neg for M07 (pos for M36)	Neg	Neg

Cells filled in grey indicate that the substance was fully outside of the applicability domain for the relevant tool and hence unreliable.
Cells with red text indicate that the substance was partially outside of the applicability domain for the relevant tool and hence only partially reliable.

The predictions for the selected metabolites and their conjugates are largely similar to those for the parent compound.

Ames
Negative predictions for bacterial mutagenicity were obtained for the active compound, all selected metabolites and their conjugates from models within Derek and Topkat. All substances were fully or partially inside the applicability domain for these two tools; hence the reliability of these predictions is acceptable. This is further supported by the fact that the negative predictions for the active substance have been confirmed by test data. Two separate negative Ames predictions were obtained for the active substance, its selected metabolites and all conjugates from the Leadscope database; however only the predictions for M02 and M11 were inside the applicability domain. Four Ames models were investigated within the Vega suite of tools. Of these, the ISS model gave a negative prediction for all substances, the KNN/read-across model gave a negative prediction for all substances except M36 (a conjugate of M07) and the CAESAR model gave a negative prediction for all substances except the metabolites M01 and M02 (although the predictions for the conjugates of these two metabolites were negative). All substances were outside of the applicability domain for the models within the Vega suite of tools and so therefore the reliability of these predictions is somewhat limited. Reliable negative predictions were reported for the parent and the selected metabolites by Toxtree. Overall, in relation to bacterial mutagenicity, reliable negative predictions were obtained for the selected metabolites from three models, Derek, Topkat and Toxtree.

Mammalian cell gene mutation
Negative predictions for mammalian gene mutation were obtained from several models within the Leadscope suite of tools (for all substances), but none were within the applicability domain. These predictions for mammalian cell gene mutations are therefore unreliable. However, no genotoxicity alerts were fired for the active compound, all selected metabolites and their conjugates from Derek.

In vitro and in vivo clastogenicity
Within the Leadscope of tools, negative predictions for *in vitro* clastogenicity were provided by three models (Chrom Ab CHO v.2, SCE CHO v.1 and SCE Comp v.1) for all substances; these were inside the applicability domain for M02 and M07 only. A further model in Leadscope gave a negative prediction for this end-point for isoflucypram, M01 and M06 but gave a positive prediction for M02, M07 and M11; the coverage of isoflucypram and its metabolites by the applicability domains within Leadscope is limited, hence the reliability of the predictions is somewhat reduced. Overall, no clear, reliable predictions for the selected metabolites in relation to in vitro clastogenicity can be derived. Negative predictions with regard to in vivo clastogenicity

were obtained from all models within Leadscope, but again all predictions were outside the applicability domains. Therefore, no reliability can be attached to these predictions for in vivo clastogenicity. However, no genotoxicity alerts were fired for the active compound, all selected metabolites and their conjugates from Derek.

In vivo micronucleus
An alert was triggered for all substances for the *in vivo* micronucleus model within Toxtree, which indicated that all substances, including isoflucypram, were H-acceptors. Although this alert occurred within the applicability domain, the reliability of this model is limited, with only 34% true positive predictions in external reliability tests. This is further supported by the negative result of the in vivo micronucleus study with the parent. In addition, no genotoxicity alerts were fired for the active compound, all selected metabolites and their conjugates from Derek. Overall, no reliable predictions for in vivo micronuclei can be obtained.

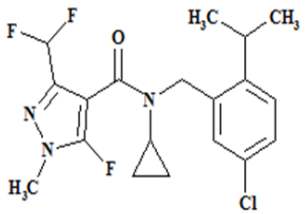
Conclusion
Overall, where reliable predictions (bacterial mutagenicity in several models and other genotoxicity alerts from Derek) were obtained, these were negative. Therefore, based on these predictions, none of the metabolites or their conjugates are expected to be genotoxic. Therefore, no genotoxicity testing of the selected metabolites is proposed.

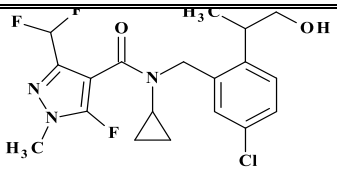
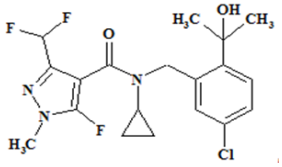
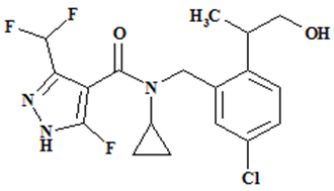
Overall toxicological assessment of selected metabolites

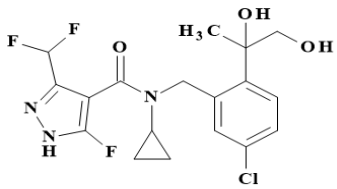
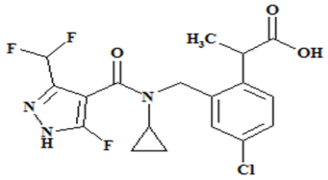
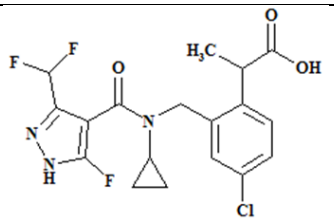
The table below summarises the available toxicological data for the selected metabolites (M01, M02, M06, M07, M11 and M12). For **M01**, **M02**, **M06**, **M11** and **M12**, there are close structural similarities to the parent compound and negative genotoxicity QSAR predictions. In addition, plasma levels of these metabolites in the 2-year study in rats were similar or higher than those of the parent. Therefore the toxicity of these metabolites is covered by the toxicity data of the parent and if a risk assessment were to be required, the reference values of isoflucypram could be used.

For **M07**, there is close structural similarity to the parent compound and negative genotoxicity QSAR predictions, but no information on relative levels in rat plasma compared to the parent. Therefore, the toxicity of M07 is not sufficiently covered by the toxicity data of the parent and if a risk assessment were to be required, the Cramer class III TTC value could be used.

Table B.6.8-2 Overall toxicological assessment of selected metabolites

No.	Structure	Name/code no. (synonyms)	Rat ADME coverage (% of administered dose)	Toxicological assessment
a.s		**Isoflucypram** BCS-CN88460 ISY LYAM823-1-2	Not detected in urine, bile or plasma	Active substance Full data package available
M01		BCS-CN88460-propanol **BCS-CY24813**	Two isomeric glucuronides of M01 (M19 isomer 1 and 2) found in the bile of rats	No study data available. Hydroxylated parent; similar to parent.

	 *$C_{19}H_{21}ClF_3N_3O_2$* *[415]*		Sum of 10.45% in males, 15.73% in females (in bile)	Found in rat plasma collected after 2-years dosing with parent, at similar concentrations of parent. Toxicity of M01 covers toxicity of conjugates M18, M19 and M21. Negative genotoxicity prediction. **Overall, toxicity of M01 covered by the toxicity data of parent.**
M02	 *C19 H21 Cl F3 N3 O2* *[415]*	BCS-CN88460-2-propanol **BCS-DC20298**	Not detected in bile or urine	No study data available. Hydroxylated parent; similar to parent. Found in rat plasma collected after 2-years dosing with parent, at similar concentrations of parent. Toxicity of M02 covers toxicity of conjugates M20 and M22. Negative genotoxicity prediction. **Overall, toxicity of M02 covered by the toxicity data of parent.**
M06	 *C18 H19 Cl F3 N3 O2* *[401]*	BCS-CN88460 – desmethyl-propanol **BCS-DC22055**	Two isomeric glucuronides of M06 (M31 isomer 1 and 2) found in the bile fluid of rats. M31 isomer 1 found in co-elution peak at 13.22% in male and 11.94% in female. Sum of M06 and M31 isomer 2 found at	No study data available. Demethylated M01; similar to parent. Found in rat plasma collected after 2-years dosing with parent, at higher concentrations than parent. Toxicity of M06 covers toxicity of conjugates M37 and M41. Negative genotoxicity prediction. **Overall, toxicity of M06 covered by the**

			1.25% in males, 5.70% in females (in bile)	**toxicity data of parent.**
M07	 *C18 H19 Cl F3 N3 O3 [417]*	BCS-CN88460-desmethyl-1,2-propandiol **BCS-CN88460**	Not detected in urine, bile or plasma	No study data available. Hydroxylated M06; similar to parent. Toxicity of M07 covers toxicity of conjugate M36. Negative genotoxicity prediction. **Overall, toxicity of M07 is not covered by the toxicity data of parent. Cramer class III TTC value could be used if required.**
M11	 *C18 H17 Cl F3 N3 O3 [415]*	BCS-CN88460-desmethyl-carboxylic acid **BCS-CX99799** ROI 5	In bile and urine, max. 4.37% in urine.	No study data available. Demethylated M12; similar to parent. Found in plasma of toxicological studies with parent, at higher concentrations than parent. Negative genotoxicity prediction. **Overall, toxicity of M11 covered by the toxicity data of parent.**
M12	 *C18 H17 Cl F3 N3 O3 [415]*	BCS-CN88460-carboxylic acid **BCS-CY26497** MXM 7275-1-5 ROI 1 M12	In the bile: female: 1.35% male: 2.48% In urine: female: 1.14% (high dose)	No study data available. Oxidized M01; similar to parent. Found in rat plasma collected after 2-years dosing with parent, at similar concentrations of parent. Negative genotoxicity prediction. **Overall, toxicity of M12 covered by the**

				toxicity data of parent.

B.6.8.1.2. Toxicity studies on relevant impurities

The impurity BCS-CN45153 is considered to be a relevant impurity because some adverse effects were detected during its development as a potential active substance [development was halted early as a result of these adverse effects]. Consequently, three studies conducted on the impurity BCS-CN45153, comprising two 28-day dietary studies (one conducted in rats and one in mice) and a uterotrophic assay in immature female rats are available for evaluation.

B.6.8.1.2.1. 28-day study in rats

Study	Exploratory 28-day toxicity study in the rat by dietary administration
Reference	[redacted] 2011
Test facility	[redacted]
Report reference	SA 10158
Guideline(s)	None
Deviations from the guideline	N/A
GLP	No
Test material	BCS-CN45153
Study acceptable	Yes

A full report of this study is not available owing to the development of BCS-CN45153 being halted shortly after the study was completed; a short summary of the study was submitted and is summarised below.

Methods

The isoflucypram impurity BCS-CN4513 was administered to male and female Wistar rats (4/sex/group) via the diet. Doses of 0, 200, 600 and 1800 ppm corresponded to estimated mean intakes of 0, 17.3, 50 and 152 mg/kg bw/d in males and 0, 17.6, 54 and 145 mg kg bw/d in females.

Results

There were no deaths or treatment-related clinical signs of toxicity. In high-dose females (145 mg/kg bw/d) a body-weight loss of 1g during the first week (compared with a gain of 22g in the controls) resulted in lower body weights of these animals throughout the study that were 7-12% lower than controls. In males at this dose (152 mg/kg bw/d) body weight was only affected during the first week of the study, during which body weight was 6% lower than controls and body-weight gain 25% lower. Food consumption was not affected in males; however, in females mean food consumption was reduced (in comparison with controls) by 35%, 13% and 27% during weeks one, two and three of the study. Consistent with the later studies conducted on the parent compound, the liver was a target organ in male and female rats and the kidneys were affected in male rats. Mean absolute and relative liver weights were 21% and 22% greater than controls in males and were also stated to be increased in females, although the magnitude of the increase is not reported. There were no histopathological findings and no correlation was observed with an induction of xenobiotic metabolising enzymes, which were increased in males (in comparison with controls) by 2-fold (PROD), 3-fold (BROD) and 1.3-fold (total P450). In females BROD was increased 4-fold and total ctytochrome P450 was increased by 1.3-fold. The increase in absolute and relative kidney weights in high-dose male rats was associated with an increased incidence/severity of hyaline droplets with signs of nephropathy, attributed to the accumulation of alpha-globulin and hence not relevant to humans. In the thyroid of males at the high-dose, treatment-related minimal follicular cell hypertrophy was noted.

In the uterus, minimal squamous metaplasia was noted in the endometrial glands of 2/5 high-dose females; however, there was no effect on the oestrous cycle when compared with controls.

Discussion and conclusion

Dietary administration of BCS-CN45153 to rats for 28-days resulted in reduced body weight and body-weight gain as well as increased absolute and relative weights at the LOAEL of 1800 ppm (152 and 145 mg/kg bw/d in males and females respectively); also at this dose, minimal squamous metaplasia was noted in the endometrial

glands in the uterus of 2/5 high-dose females. There were no effects at the NOAEL of 600 ppm (50/54 mg/kg bw/d in males/females).

(, 2011)

B.6.8.1.2.2. 28-day study in mice

Study	Exploratory 28-day toxicity study in the rat by dietary administration
Reference	[redacted] 2010
Test facility	[redacted]
Report reference	SA 09400
Guideline(s)	None
Deviations from the guideline	N/A
GLP	No
Test material	BCS-CN45153, batch: NLL 8224-6-4, purity: 97.6%
Study acceptable	Yes

Methods

The impurity BCS-CN45153 at doses of 0, 400, 2000 and 4500 ppm, was administered via the diet to 5/sex/group C57BL/6J mice for at least 28-days. These doses equated to estimated intakes of 0, 64, 337 and 779 mg/kg bw/d in males and 0, 73, 377 and 819 mg/kg bw/d in females.

Results

There were no deaths or treatment-related clinical signs of toxicity. At the high-dose, treatment-related alterations to body-weight development were noted in both sexes which resulted in an overall body weight-gain value at the end of the study that was lower than controls in females only (-38%); a slight decrease in food consumption was noted in these females from study day 8. Similarly to studies conducted with the parent compound, the liver was affected after administration of BCS-CN4153. The only clinical chemistry findings were in relation to levels of bilirubin (reduced in males) and cholesterol (increased in females). In females absolute liver weights were increased by 15% and 32% at 2000 and 4500 ppm respectively, whilst relative weights at these doses were increased by 17% and 38%. In males absolute liver weights were 19% and 37% greater than controls and relative weights were 23% and 42% greater at the mid- and high-doses respectively. Macroscopic examination revealed an enlarged liver in 3/5 males and 1/5 females at 4500 ppm whilst microscopic changes in the liver comprised centrilobular to panlobular hypertrophy at the high-dose in 5/5 males (slight) and 4/5 females (3 minimal and 1 slight) and also at the mid-dose in 3/5 males (2 minimal and 1 slight) and 2/5 females (minimal). Hepatocellular single cell necrosis was also observed in 3/5 high-dose females (2 minimal and one slight) and in 4/5 high-dose males (3 minimal and one slight); single incidences of this finding were also noted in the control group (males and females) and the mid-dose (males), all to a minimal extent.

Discussion and conclusion

Aside from some reductions in body-weight, only the liver was affected after dietary admnistration of BCS-CN45153 to mice. At the LOAEL of 2000 ppm (377 mg/kg bw/d), absolute and relative liver weights were increased, accompanied by an enlargment of the organ and histopathological and clinical chemistry findings. There were no adverse effects observed at the NOAEL of 400 ppm (equivalent to 64 and 73 mg/kg bw/d in males and females respectively).

([redacted] 2010)

B.6.8.1.2.3. Rat uterotrophic assay

Study	BCS-CN45153 Evaluation in the immature rat uterotrophic assay coupled with vaginal opening
Reference	[redacted] 2011
Test facility	[redacted]
Report reference	SA 10312
Guideline(s)	None
Deviations from the guideline	N/A

GLP	No
Test material	BCS-CN45153
Study acceptable	Yes

Methods

Immature female Sprague-Dawley rats (6/dose) were administered the isoflucypram impurity BCS-CN45153 via oral gavage at doses of 0, 150 and 450 mg/kg bw/d (doses were selected based on body weight reductions of 10% and 14% observed at the top-dose of 500 mg/kg bw/d in a preliminary study). Dosing continued for at least 20 days, with animals being sacrificed 24-hours after the last administered dose and the uterine weight (wet and blotted) was recoreded. Vaginal opening was recorded daily from day 10 until day 21

Results

There were no deaths; clinical signs comprised increased salivation in all treated animals and reduced motor activity and lack of grooming in 2 animals administered 450 mg/kg bw/d. There was no effect on body weight or body-weight gain. The mean age of first vaginal opening was reduced at 450 mg/kg bw/d compared with controls and was correlated to a lower mean body weight at vaginal opening. There was a slight delay in vaginal opening at the mid-dose of 150 mg/kg bw/d but this was attributed to a slight decrease in body weight as the body weights of the control animals and this dose groups were comparable at the time of vaginal opening. The mean age and body-weights at the time of vaginal opening are summarised in the table below.

Table B.6.8-3: Mean age and body weight at the vaginal opening in female rats administered impurity BCS-CN45153 for 20 days

Dose-levels (mg/kg bw/d)	**BCS-CN45153**		
	0	**150**	**450**
Mean age, days	33.5	36.0	≤ 30.3
Mean body weight, g	110.0	114.8	≤ 87.9

There was no treatment-related effect observed on uterine weights; a decrease in uterine weight observed at the mid-dose of 150 mg/kg bw/d was not considered to be related to treatment, as the change was not statistically significant and there was no dose response relationship.

Discussion and conclusion

When impurity BCS-CN45153 was administered to female rats for 20 days, there were treatment-related clinical signs at the high-dose of 450 mg/kg bw/d which comprised increased salivation, reduced motor activity and lack of grooming. The mean age and body-weight at first vaginal opening of females at this dose was statistically significantly reduced in comparison with controls. There was no effect on uterine weight at any dose.

Overall, the results show that interferes with pubertal development in the female rat at the LOAEL of 450 mg/kg bw/d; there were no effects seen at the NOAEL of 150 mg/kg bw/d.

([redacted] *2011)*

Conclusion on relevant impurity BCS-CN45153

This impurity was investigated in two 28-day studies (one in rats and one in mice) and in a uterotrophic assay in rats. Liver effects were seen in rats (at top dose of 150 mg/kg bw/d) and mice (at ≥ 377 mg/kg bw/d) and histopathological findings of the uterus were noted in rats (at 150 mg/kg bw/d). In the uterotrophic assay, an acceleration of vaginal opening was noted at 450 mg/kg bw/d. Similar effects on vaginal opening and uterus were not seen with isoflucypram. Therefore, on the basis of these effects, impurity BCS-CN45153 is considered to be a relevant impurity.

B.6.8.2. Supplementary studies on the active substance

Mechanistic studies investigating liver and thyroid effects

In the repeated-dose toxicity studies conducted in rats (see section B.6.3), isoflucypram was seen to affect the liver in both sexes (increased weight, enlargement and hepatocellular hypertrophy) and the thyroid in males (increased weight, follicular cell hypertrophy and colloid alteration) after both 28- and 90-days' exposure. It is proposed that these changes are a consequence of enzyme induction in the liver. Liver enzyme induction analysis, carried out during the 28-day rat study(see repeated-dose section), provided evidence that isoflucypram is a potential activator

of the CAR and/or PXR receptors. To further characterise this hypothesised MoA, a series of non-guideline mechanistic studies have been conducted in the rat (one 7-day and two 28-day studies). A 7-day study in the mouse has also been conducted (increased liver weights were observed in mice in the repeated-dose toxicity studies, but no alterations to thyroid parameters). All studies were conducted on female animals.

Seven-day dietary study in rats (liver and thyroid hormone measurements)

A seven-day dietary study was conducted in rats, the aim of which was to examine the effect of isoflucypram on hepatocellular and thyroid follicular cell proliferation.

Study	7-day dietary study of liver and thyroid cell proliferation in the female rat
Reference	[redacted]2018a
Test facility	[redacted]
Report reference	SA 15054
Guideline(s)	None
Deviations from the guideline	N/A
GLP	No
Test material	Isoflucypram ; batch 2013-006492 ; purity 94.2%
Study acceptable	Yes

Methods

Isoflucypram was administered to female Wistar rats (12/group) for a minimum of seven days at dietary concentrations of 0, 30, 75, 150, 450 and 800 ppm (equating to 2.4, 6, 12, 36 and 67 mg/kg bw/d). The stability of the test substance in the diet has been determined in a separate study with a validated method of analysis. An additional twelve animals were added to the control and high-dose groups and continued to receive untreated diet for a further month; this recovery group enabled the assessment of reversibity of any signs observed during the initial seven-day treatement period. All animals were observed for mortality and clinical signs (daily) as well as body weight, food consumption and clinical signs (weekly). At necropsy blood was collected for clinical chemistry and hormone evaluations and the liver, thyroid and pituitary gland were collected for further examination. Microsomal preparations of the liver were further examined to determine P450 and UDGPT isoenzyme profiles.

Results

There were no deaths or treatment-related clinical signs of toxicity. There were no changes observed with regard to body-weight development or food consumption, either during the treatment phase or in recovery. Measurement of thyroid hormones (TSH, T3 and T4) at the seven-day necropsy revealed an 85% increase in thyroid stimulating hormone (TSH) at 800 ppm (67 mg/kg bw/d) relative to controls. The applicant does not consider this to be a treatment-related effect, as the change was not statistically significant and showed no clear dose-response (TSH measurements at day 8 were 33%, 50%, 20%, 32% and 85% greater than controls in the 30, 75, 150, 450 and 800 ppm dose groups respectively). However, as increased TSH is a consistent finding in both the succeeding 28-day studies in rats, the RMS disagrees with this conclusion and considers that the effect at least at the top dose of 67 mg/kg bw/d is related to treatment with isoflucypram. Slight increases in T3 at 450 ppm (36 mg/kg bw/d) and 150 ppm (12 mg/kg bw/d) of 23%* and 34%** respectively were not considered to be treatment-related by the RMS owing to the lack of a dose-response. Furthermore, a slight increase in T3 at 800 ppm (recovery phase) was not related to treatment as the small magnitude of the response (26%) means that this is more likely to be an incidental finding. Table B.6.8-4 summarises the findings of the thyroid hormone analysis after 7-days (main groups) and 28-days (recovery groups).

Table B.6.8-4: Concentrations of circulating thyroid hormones in female rats administered isoflucypram for 7 days

Dose-levels (ppm)	**BCS-CN88460, dietary concentration in ppm**						
	Hormone	**0**	**30**	**75**	**150**	**450**	**800**
Day 8	T3	0.47	0.49 (4%)	0.52 (11%)	0.63** (34%)	0.58* (23%)	0.53 (13%)
	T4	2.59	2.51	3.09	2.35	2.70	2.33 (-10%)
	TSH	0.820	1.092 (33%)	1.228 (50%)	0.978 (20%)	1.081 (32%)	1.523 (85%)

Dose-levels (ppm)	BCS-CN88460, dietary concentration in ppm						
	Hormone	**0**	**30**	**75**	**150**	**450**	**800**
Day 29 (recovery)	T3	0.50	-	-	-	-	0.63** (26%)
	T4	2.76	-	-	-	-	2.83 (2.5%)
	TSH	1.064	-	-	-	-	0.851 (-20%)

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Relative liver weights (to body weight and brain weight) were statistically significantly higher than controls at 800ppm (67 mg/kg bw/d) by 9% and 12% respectively. The RMS considers this effect to be related to treatment with isoflucypram, but not adverse. The weight of the thyroid was not affected by treatment with isoflucypram. The increased liver weights at 800 ppm during the treatment phase were associated with an enlargement of the organ in 6/12 animals, one of which persisted to the end of recovery.

Cell proliferation has been evaluated in the liver and the thyroid by immunohistochemical staining of paraffin embedded sections, for the detection of Ki67 nuclear protein (and the subsequent determination of the labelling index). Total mean profilerative indices in both these organs at 800 ppm were statistically significantly higher than controls in a broadly dose-related manner (see table B.6.8-5 below); these were found to be reversible effects because during the recovery phase at 800 ppm, mean proliferative indices were lower than controls in the liver and comparable to controls in the thyroid.

Table B.6.8-5: Cell proliferation in the liver and thyroid of female rats after treatment with isoflucypram for 7 days

		Dietary concentration in ppm					
Organ	**Location**	**0**	**30**	**75**	**150**	**450**	**800**
Treatment phase							
Liver	Centrilobular	20.16	20.54 (+2%)	25.41 (+26%)	30.01 (+49%)	30.03 (+49%)	37.19 (+85%)
	Periportal	34.28	25.50 (-26%)	37.32 (+9%)	38.13 (+11%)	48.00 (+40%)	67.79** (+98%)
	Total	27.22	23.02 (-15%)	31.37 (+15%)	34.07 (+25%)	39.02 (+43%)	52.50** (+93%)
Thyroid		23.65	22.53	31.23	25.24	37.74	49.97*** (+111%)
Recovery phase							
Liver	Centrilobular	5.96	-	-	-	-	2.99 (-50%)
	Periportal	12.72	-	-	-	-	4.63*** (-64%)
	Total	9.34	-	-	-	-	3.81*** (-59%)
Thyroid		11.85	-	-	-	-	13.05 (+10%)

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Analysis of phase I hepatic enzymes revealed that the largest increase was in PROD (a substrate for CYP 2B and an indicator of CAR activation); the increase (65% at 800 ppm) was generally dose-related, although it was not statistically significant. Total P450 was also increased at this dose but by a smaller magnitude (19%) and the remaining phase I enzymes, BROD and EROD (substrates for CYP1A and CYP3A, indicative of AhR and PxR activation) were slightly increased at 800 ppm; however the changes were of a small magnitude, were not statistically significant and showed no evidence of a dose-response. With regard to phase II enzymes, increases in the UDPGT enzymes acting on bilirubin, T4 and 4-nitrophenol were increased by 169%, 131% and 20% respectively. No treatment-related increases in hepatic enzyme activity were noted during the recovery phase, suggesting that there is the potential for reversibility of the effects seen during treatment.

Table B.6.8-6: Activities of Phase I and II enzymes after dietary administration of isoflucypram for 7 days

Dose-levels (ppm)	Females					
	0	**30**	**75**	**150**	**450**	**800**
Treatment phase						
Cytochrome P450 nmol/mg protein	0.882	0.815	0.839	0.917	0.893	1.048**
% change	-			+4%	+1.2%	+19%
BROD Pmol/min/mg protein	3.873	3.583	3.421	3.844	6.809	5.444
% change						40.5%
EROD Pmol/min/mg protein	21.371	27.788	31.908**	25.095	23.578	26.288
% change						23%
PROD Pmol/min/mg protein	1.548	1.763	2.238	1.998	2.270	2.548
% change	-	+14%	+44.6%	+29%	+46.6%	+65%
p-nitrophenol-UDPGT Nmol/min/mg protein	6.495	5.868	5.945	6.427	6.950	7.797**
% change	-	-9.7%	-8.5%	-1%	7%	20%
Bilrubin-UDPGT Nmol/min/mg protein	0.5543	0.3913	0.4566	0.5378	0.9764*	1.4886***
% change	-	-29.4%	-17.6%	-3%	+76%	+168%
T4-UDPGT Pmol/min/mg protein	0.492	0.500	0.751	0.710	0.821	1.136**
% change	-	+1.6%	+52.6%	+44.3%	+66.8%	+131%

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Analysis of gene transcription, revealed dose-related increases of genes related to the phase I and II enzymes dependent on CAR/PXR receptors (CYP2 B1), during treatment but not the recovery phases (see table B.6.8-7 below). Phase I enzymes dependent upon other receptors such as AhR (CYP1 A1/A2) and PPAR (CYP4 A1) did not show any increase in transcription.

Table B.6.8-7: Gene transcription in female rats administered isoflucypram for 7 days

	Females					
Dose–levels (ppm)	**0**	**30**	**75**	**150**	**450**	**800**
Treatment phase						
Cyp1a1	1.0000	0.3017	1.1050	0.9483	1.3242	0.8042
Cyp1a2	1.0000	0.8383	0.9350	1.0458	1.0242	0.8400
Cyp2b1	**1.0008**	**0.8817**	**4.6317****	**6.9317****	**42.8017****	**71.2533****
Cyp4a1	1.0008	1.0450	1.0900	0.9983	0.9433	0.7733*
Ugt1a1	1.0000	0.9167	1.0992	1.3550**	2.9075**	4.2925**
Ugt1a6	0.9992	0.9433	0.9325	1.1167	1.3300**	1.4208**
Ugt2b1	0.9992	1.3475	1.7900*	1.7158*	2.5583**	3.7033**
TSHβ	0.9691	1.2250	1.2155	1.0875	1.3867	1.480
Reversibility phase						
Cyp1a1	0.9991					1.3217
Cyp1a2	1.0009					1.1617
Cyp2b1	1.0000					1.3842
Cyp4a1	1.0000					1.0183
Ugt1a1	1.0000					0.9325
Ugt1a6	1.0000					0.9550
Ugt2b1	1.0000					0.9925
TSHβ	0.9992					1.0150

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Discussion and Conclusion

Dietary administration of isoflucypram for 7 days to Wistar rats resulted in slight increases in relative liver (but not thyroid) weights of females at the top dose of 800 ppm (67 mg/kg bw/d). Cell proliferation was noted in the

thyroid and in the liver at the top dose. TSH was increased at 800 ppm (67 mg/kg bw/d) by 85% relative to controls. The induction of phase I and II enzymes associated with CAR activation (and to a lesser extent with PXR activation) and observed from 75 ppm (6 mg/kg bw/d) was demonstrated, as well as an increase in the transcription of their associated genes.

Overall, this short mechanistic study demonstrates that in female rats, CAR activation is a likely mechanism for the effects seen in the liver and thyroid in the repeated-dose studies, whilst PXR activation is possible and the activation of AhR or PPAR is unlikely according to the findings of this study. This suggests that isoflucypram possibly works via a phenobarbitol-like mechanism involving CAR activation.

([redacted] 2018a)

28-day dietary study in the rat (with liver enzyme induction and thyroid hormones)

A 28-day dietary mechanistic study was conducted in rats to investigate the histopathological changes in the liver and thyroid (noted in the repeated-dose toxicity studies), by characterising the phase I and II hepatic enzyme induction profile and circulating thyroid hormone levels.

Study	BCS-CN88460 – machanistic 28-day toxicity study in the female rat by dietary administration (hepatotoxicity and thyroid hormone investigations)
Reference	[redacted] 2013
Test facility	[redacted]
Report reference	SA 12190
Guideline(s)	None
Deviations from the guideline	N/A
GLP	No
Test material	Isoflucypram ; batch NLL 8674-19-4 ; purity 98.6%
Study acceptable	Yes

Methods

Based on the findings of the previous 28-day repeated-dose toxicity study in rats (see section B.6.3.1.1), groups of 10 female rats received doses of isopyflucypram that equated to 0, 26 and 85 mg/kg bw/d (0, 300 and 1000 ppm) in the diet for at least 28 days. All animals were observed for mortality and clinical signs daily, whilst body-weight and food consumption were recorded weekly. At necropsy blood was collected for thyroid hormone measurements. Gene transcript analysis was conducted on portions of the liver and pituitary from each animal and phase I and II isoenzyme profiles were determined from homogenised microsomal liver preparations.

Results

There were no deaths or clinical signs of toxicity. Overall body-weight gain was slightly reduced at 1000 ppm (85 mg/kg bw/d) but the change was not statistically significant; there was no effect on body-weight gain at 300 ppm (26 mg/kg bw/d) nor on mean body weight at either dose. Food consumption was reduced at 85 mg/kg bw/d for the duration of the study but statistical significance was only reached during the first week. Thyroid hormone measurements taken at the end of the study revealed a dose related increase in TSH in both treated groups, although only at 1000 ppm was statistical significance reached (see table B.6.8-8 below). The RMS considers the increase in TSH to be treatment-related; there was no effect on the levels of T3 or T4 at either dose.

Table B.6.8-8: Concentrations of thyroid hormones in female rats administered isoflucypram for 28 days

Dose-levels (ppm)	**Females**		
	0	**300**	**1000**
T3	0.90	0.97	0.94
T4	2.36	2.31	2.13
TSH	0.381	0.586	1.018**
% change	-	+53.8%	+167%

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Only the liver was weighed; at 1000 ppm (85 mg/kg bw/d) absolute and relative liver weights were statistically significantly increased in comparison with controls by 18.9% and 18.2% respectively. At 300 ppm (26 mg/kg bw/d) absolute and relative liver weights were 8.7% and 8.2% greater than controls respectively, although only the increase in absolute weight was statistically significant (see table B.6.8-9 below).

Table B.6.8-9: Liver weight increases in female rats administered isoflucypram for 28 days

Dose levels (ppm)	Females		
	0	300	1000
Absolute liver wt, g	8.234	8.951	9.794**
% change	-	8.7%	18.9%
Liver wt, % body wt	3.269	3.54*	3.865**
% change	-	8.3%	18.2%

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

There were no macroscopic findings and the collected tissue (liver) was not examined microscopically. Enzyme induction investigations revealed a dose-related increase in the activity of BROD, bilirubin-UDGPT and T4 UDGTP (see table B.6.8-10 below). The activity of PROD was reduced at the high-dose only.

Table B.6.8-10: Activity of specific hepatic P450 isoenzymes in female rats administerd isoflucypram for 28 days

Dose-levels (ppm)	Females		
	0	300	1000
BROD Pmol/min/mg protein	4.34	5.49*	6.83**
% change	-	26%	57%
PROD Pmol/min/mg protein	3.45	3.68	2.74*
Bilirubin-UDPGT Nmol/min/mg protein	0.35	0.81*	2.08***
% change		131%	494%
T4-UDPGT Pmol/min/mg protein	1.03	2.09*	4.37**
% change		102%	324%

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

The transcription of several phase I and II genes including those which code for P450 1A1, 2B1 and 3A3 was increased. The increment in the transcription of cyp 1A1 is regarded as a spurious finding, as no induction of EROD was observed in the subsequent 28-day mechanistic study in rats, which followed a similar protocol as the present investigation (see section B.6.8.2.3 below). Although cyp 2A1 was found to be increased there was no corresponding increase in the activity of PROD (such as that seen in the previous 7-day study); therefore it is possible that the increase in cyp 2A1 is also not related to treatment. Other findings after qPCR analysis include the transcription of P450 oxyreductase (Por) indicative of an overall up-regulation of P450 activity; the accumulation of THSβ transcript in the pituitary was also increased in treated groups (see table B.6.8-11 below).

Table B.6.8-11: Gene transcription for for hepatic genes and TSH in female rats administered isoflucypram for 28 days

Dose-levels (ppm)	Females		
	0	300	1000
Cyp1a1	1.88	2.00	10.50**
Cyp2b1	0.25	6.61	31.10**
Cyp3a3	0.77	20.87	98.27***
Cyp4a1	0.96	0.88	0.75*
Por	0.56	0.85**	1.35**
Ugt1a6	0.69	0.91	2.16***
Ugt2b1	0.65	1.15**	2.39**
Sult2a2	0.67	1.14	1.31*
Ephx1	0.72	1.53**	2.85**
Gstm4	0.48	1.83**	4.41**
TSHβ	1.09	1.26	1.87*

Discussion and conclusion

Dietary administration of isoflucypram to femal rats for 28 days, resulted in absolute and relative liver weight increases at 1000 ppm (85 mg/kg bw/d) of approximately 18%. TSH was increased by 167% at this dose (in comparison with controls) and the enzyme induction profile and gene transcription analysis revealed treatment-

related increases in BROD (by 57%) and the associated genes (cyp 3a3; increased by 98.27%) from 300 ppm (26 mg/kg bw/d). This suggests that isoflucypram is working via a PXR MoA according to this study. The transcription of genes associated with CAR was also increased (cyp 2B1; increased by 31.10%) but there was no resultant increase detected in the activity of PROD; therefore this study suggests that, contrary to the previous seven-day study in rats, PXR activation is occurring to a greater extent than CAR activation.

([redacted] *2013)*

28-day dietary study in the rat (liver and thyroid cell proliferation).

A 28-day mechanistic dietary study was conducted in female rats to further assess the cell proliferation observed in the liver and thyroid of rats in the previously conducted guideline 28-day repeated-dose toxicity study in rats (2017).

Study	BCS-CN88460 – 28-day mechanistic toxicity study for liver and thyroid cell proliferation in female wistar rats
Reference	[redacted] 2018b
Test facility	[redacted]
Report reference	SA 15258
Guideline(s)	None
Deviations from the guideline	N/A
GLP	No
Test material	Isoflucypram ; batch 2013-006492 ; purity 94.2%
Study acceptable	Yes

Methods

Isoflucypram was administered to the diet of Wistar rats (12/dose) at concentrations of 0, 30, 75, 150, 450 and 800 ppm (corresponding to 0, 2.4, 6, 12, 37 and 69 mg/kg bw/d) for 28-days. A recovery group comprised 12 additional females in the control and high-dose groups which were fed untreated diet for a further month after treatment; this enabled the assessment of the potential for reversibility of any effects seen during the study. Mortality and clinical signs were measured daily and body-weight and food consumption were recorded weekly. Blood was collected prior to necropsy for hormone and clinical chemistry analysis. The liver, thyroid and pituitary gland were collected for necropsy. Gene expression investigations were carried out on portions of the liver and pituitary and the remaining liver portions were homogenised for microsomal preparations (for P450 and UDPGT isoenzyme profiling).

Results

There were no deaths or clinical signs of toxicity and there were no changes to body weight parameters or food consumption. Consistent with other repeated-dose studies with isoflucypram, a dose-related decrease in bilirubin was noted which did not persist into the recovery phase.

Thyroid hormones were measured at necropsy and a dose-related increase in TSH was evident, which reached statistical significance at 800 pmm /69 mg/kg bw/d (97% greater than controls); there were no statistically significant changes to TSH in the recovery groups, or of T3 or T4 in either the main or the recovery groups (see table B.6.8-12 below).

Table B.6.8-12: Thyroid hormone concentrations in female rats administered isoflucypram for 28 days

Dose-levels (ppm)	**Females**					
	0	**30**	**75**	**150**	**450**	**800**
Treatment-phase animals						
T3 ng/mL	0.41	0.39	0.40	0.44	0.41	0.37
T4 µg/dL	2.21	2.24	2.17	2.49	2.64	2.27
TSH ng/ml	0.720	0.734	0.857	0.882	0.942	1.421**
% change in TSH	-	1.9%	+19%	+22.5%	+30.83%	+97.36%
Reversibility group						
T3 ng/mL	0.44	-	-	-	-	0.41
T4 µg/dL	2.25	-	-	-	-	2.25
TSH ng/ml	0.612	-	-	-	-	0.862

Significant at * $p \leq 0.05$; ** $p \leq 0.01$;

Table B.6.8.2.10 below summarises the treatment-related organ weight changes. Absolute and relative liver weights were increased in comparison with controls at 800 ppm by 17%. and 18% respectively; the increase was dose-related from 75 ppm (an increase at 30 ppm was considered a spurious finding owing to a lack of a dose-response). The changes in liver weight were not present in the recovery phase and isoflucypram administration did not have any effect on thyroid weights.

Table B.6.8-13: Selected organ weight changes (liver) in female rats administered isoflucypram for 28 days

Dose-levels (ppm)	Females					
	0	**30**	**75**	**150**	**450**	**800**
Treatment-phase animals						
Liver weight (g)	8.380	9.612**	8.777	9.049	9.125	9.839***
% change	-	14.7**	4.7%	8%	8.9%	17.4%***
Relative liver weight (% of body weight)	3.053	3.375**	3.154	3.212	3.280*	3.611**
% change	-	10.5%	3.3%	5.2%	7.4%	18.3%
Reversibility group						
Liver weight (g)	8.349	-	-	-	-	8.764
Relative liver weight (% of body weight)	2.768	-	-	-	-	2.924

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

An enlarged liver was noted in 10/12 animals at 800 ppm, which for two of these persisted into recovery; the livers of 6/12 rats at 30 ppm were also enlarged, reflecting the spurious increased weight of the organ at this dose. Histopathological correlates (hepatocellular hypertrophy) were noted at the high-dose. In the thyroid, there were no macroscopic findings but follicular cell hypertrophy was seen in 50% of the high-dose group which had recovered by the end of the additional phase (see table B.6.8-14 below).

Table B.6.8-14: Macroscopic/microscopic findings (liver and thyroid) in female rats administered isoflucypram for 28 days

Dose-levels (ppm)	Females					
	0	**30**	**75**	**150**	**450**	**800**
Treatment phase						
Liver						
Examined	12	12	12	12	12	12
Enlarged	1	6	0	1	1	10
Hepatocellular hypertrophy	0	0	0	0	0	5
Thyroid						
Follicular cell hypertrophy	0	0	0	0	0	6
Recovery phase						
Liver						
Examined	12	0	0	0	0	12
Enlarged	0					2
Hepatocellular hypertrophy	0					0
Thyroid						
Follicular cell hypertrophy	0					0

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

Cell proliferation in the liver was increased in a dose-related manner (with the exception of the spurious findings at 30 ppm), although statistical significance was only reached at the top dose of 800 ppm during the recovery phase and not the main study. In the thyroid cell proliferation was increased in all treated groups (statistically significant at 450 and 800 ppm); the changes in the thyroid were not seen in the recovery phase (see table B.6.8-15 below).

Table B.6.8-15: Cell proliferation in the liver and thyroid of female rats administered isoflucypram for 28 days

Dose-levels (ppm)	Females					
	0	30	75	150	450	800
Treatment phase						
Liver						
Centrilobular	9.377	11.913	8.873	9.616	12.023	12.813
Periportal	13.562	23.635	16.112	17.314	18.206	20.768
Total	11.471	17.776	12.495	13.467	15.118	16.794
Thyroid						
Total	13.137	15.527	18.896	17.008	21.263**	23.311**
Reversibility phase						
Liver						
Centrilobular	1.913					0.528*
Periportal	6.811					2.943**
Total	4.363					1.737**
Thyroid						
Total	8.605					7.109

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

Measurment of hepatic enzyme activity did not reveal any alterations to the levels of total cytochrome P450; BROD activity was statistically significantly increased from 450 ppm whilst the activity of EROD was inversely proportionate to the increasing isoflucypram concentration. Bilirubin-UDGPT and T4-UDGPT were increased in a dose related manner with the latter being statistically significant in all treated groups and a small (non statistically significant increase was observed in PNP-UDGPT; table B.6.8-16 (below) summarises the induction of phase I and II enzymes in the main phase of the study (there was no effect on these enzymes during the recovery phase).

Table B.6.8-16: Liver enzyme induction in female rats administered isoflucypram for 28 days

Does-levels (ppm)	Females					
	0	30	75	150	450	800
Total P450 Nmol/mg protein	0.994	1.106	0.896	0.893	0.965	1.025
BROD Pmol/min/mg protein	2.716	4.173*	3.441	3.878	4.422*	6.737**
% change	-	+53.64%*	+26.69%	+42.78%	+62.81%*	+148%**
EROD Pmol/min/mg protein	33.672	29.272	27.039	27.573	26.711	20.323**
PROD Pmol/min/mg protein	4.149	4.227	4.060	4.790	4.738	4.784
PNP-UDPGT Nmol/min/mg protein	6.730	7.793	6.687	6.447	8.105*	7.940
BL-UDPGT Nmol/min/mg protein	0.7711	0.8328	0.8781	0.7789	1.5236**	1.9408**
T4-UDPGT Pmol/min/mg protein	1.070	1.787**	2.102**	1.573*	2.242**	3.255**

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

During the main phase of the study there was a clear dose-related increase in Cyp2b1, Cyp3a23, Ugt1a1 and Ugt2b1; of these only the latter persisted into the recovery phase (to a statistically significant level), and Ug1a1 was actually lower than controls during recovery. There was no effect on the transcription of Cyp4a1 and, although an increase in Cyp1a1 was observed, it was not considered to be relevant owing to the observed decrease in EROD.

Table B.6.8-17: Gene transcription in female rats administered isoflucypram for 28 days

Dose-levels (ppm)	Females					
	0	30	75	150	450	800
Treatment phase						
Cyp1a1	1.00	1.142	1.182	1.477	1.364	2.659**
Cyp1a2	0.998	0.907	1.126	0.936	0.985	1.036
Cyp2b1	0.999	2.183	3.866	10.853**	26.395**	102.785**

Dose-levels (ppm)	Females					
	0	**30**	**75**	**150**	**450**	**800**
Cyp3a23	0.993	0.800	2.061*	2.908**	12.835**	29.575**
Cyp4a1	1.001	1.022	1.207	0.907	0.962	0.750
Ugt1a1	0.999	0.946	1.355*	1.435**	2.650*	4.068**
Ugt2b1	1.000	1.253	1.106	1.436	2.351**	3.318**
TSHβ	0.999	1.062	0.961	1.039	1.028	1.188
Recovery phase						
Cyp1a1	0.999					1.921
Cyp1a2	1.000					1.090
Cyp2b1	1.001					1.271
Cyp3a23	0.994					0.798
Cyp4a1	1.000					0.894
Ugt1a1	1.000					0.857*
Ugt2b1	1.000					1.518*
TSHβ	1.007					0.924

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

Discussion and conclusion

Isoflucypram was administered via the diet to female Wistar rats for 28-days; findings were similar to those observed in the previous 28-day mechanistic study in rats which followed a similar protocol. Liver weights were increased (17% absolute and 18% relative) at the top dose of 800 ppm (69 mg/kg bw/d) and associated macroscopic (enlarged liver) and microscopic (hepatocellular hypertrophy) findings were evident at this dose. In the thyroid follicular cell hypertrophy was observed, and similarly to the previous study, TSH (but not T3 or T4) was elevated in comparison with controls at 800 ppm; also in the thyroid, follicular cell hypertrophy was noted more noticeably at 800 ppm. Analysis of cell proliferation in the liver and thyroid revealed a dose-related increase in cell proliferation in both organs which persisted into recovery in the liver only.

Analysis of liver enzyme induction again suggested that isoflucypram is most likely to be acting through a PXR activation mechanism (although CAR activation cannot be confidently excluded). Confirming PXR activation, BROD and the transcription of its associated gene (cyp 3a23) was increased. Therefore it is likely that isoflucypram is an activator of PXR based on this study.

([redacted] 2018b)

7-day dietary study in the mouse (liver enzyme induction and cell proliferation)

Study	BCS-CN88460 – Mechanistic 7-day toxicity study for liver and thyroid cell proliferation in the C57BL/6J female mouse
Reference	[redacted] 2018c
Test facility	Bayer S.A.S
Report reference	SA 14037
Guideline(s)	None
Deviations from the guideline	N/A
GLP	No
Test material	Isoflucypram ; batch 2013-006492 ; purity 94.2%
Study acceptable	Yes

Methods

Female mice of the strain C57BL/6J (15/group) received isoflucypram in the diet for at least seven days, at dose levels of 0, 50, 110, 250 and 1250 ppm; doses which equated to 0, 8.9, 19, 44, 94 and 224 mg/kg bw/d. The control and the high-dose groups contained an additional 15 mice to assess the reversibility of any observed effects; these mice were kept (untreated) for a further month after the conclusion of the seven-day treatment period. Clinical observations were carried out daily and body-weight and food consumption were measured weekly. At necropsy the liver was collected and weighed for use in several investigations.

Results

There were no deaths or clinical signs of toxicity, neither were there any effects on body-weight or food consumption throughout the study. Liver weights were increased from 44 mg/kg bw/d (250ppm) in a dose-related

manner and were accompanied by macroscopic findings (enlarged liver) but no microscopic findings; the effect did not persist into the recovery phase (see table B.6.8.2.14 below).

Table B.6.8-18: Liver weights in female mice administered isoflucypram for 7 days

Day	Dose levels (ppm)	Females					
		0	50	110	250	560	1250
8	Terminal body wt, g	19.55	19.65	19.58	20.25	19.73	19.44
	Absolute liver wt, g	0.964	1.013	1.018	1.105**	1.116**	1.216**
	% change	-	+5%	+6%	+15%**	+16%**	+26%**
	Relative liver weight (% body weight)	4.929	5.148	5.193	5.448**	5.653**	6.253**
	% change	-	+4%	+5%	+11%	+15%	+27%
	Liver, enlarged	0	1	0	3	4	8
29 (recovery)	Terminal body wt, g	21.90	-	-	-	-	21.23*
	Relative liver wt (% body weight)	5.108	-	-	-	-	5.135

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

When measured using BrdU labelling, higher centrilobular, periportal and total cell profilaration indices were observed in the liver from 250 ppm (44 mg/kg bw/d); however, there was a high variability and no clear dose-response (see table B.6.8-19 below). When assessed with Ki67 labelling, higher cell proliferation indices were observed at 250 and 560 ppm but not at 1250 ppm, again with no clear dose-response. Therefore owing to the high variability and lack of a dose-response no conclusion on liver cell proliferation in mice could be made. No treatment related effects on cell proliferation in the thyroid were observed with either BrdU or Ki67 labelling.

Table B.6.8-19: Labeling indices obtained with BrDU or Ki67 in livers of female mice administered isoflucypram for 7 days

Dose-levels (ppm)	Females					
	0	50	110	250	560	1250
BrDU labelling index						
Centrilobular	39.97	43.74	61.56	116.56*	77.08	66.63
Periportal	36.64	38.18	57.07	102.12**	90.39*	94.62
Total	38.31	40.96	59.31	109.34**	83.74	80.62
Ki67 labelling index						
Centrilobular	14.19	7.66	14.17	26.37	22.43	11.78
Periportal	9.50	7.32	10.68	19.27	19.53	17.26
Total	11.85	7.49	12.43	22.82	20.98	14.52

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

Total cytochrome P450 content, specific P-450 enzyme activity and UGT-BL specific isoenzyme profiles were determined from the homogenised microsomal preparation of the livers of 10 mice per dose group, for both the treatment and the recovery phases. Total P450 content was increased from 560 ppm in a dose –related manner and increases were seen in the activities of PROD at all doses, BQ from 560 ppm and bilirubin-UDPGT from 250 ppm; none persisted into the recovery phase, thus indicating total reversibility of liver enzyme induction (see table B.6.8-20 below).

Table B.6.8-20: Hepatic phase I and II enzyme induction in female mice administered isoflucypram for 7 days

Dose-levels (ppm)	Females					
	0	50	110	250	560	1250
Treatment phase						
Cytochrome P450 nmol/mg protein	0.30	0.28	0.36	0.35	0.47**	0.55**
PROD Pmol/min/mg protein	11.18	16.16**	19.52**	32.80**	60.09**	128.89**
BQ Nmol/min/mg protein	4.39	4.29	4.03	4.94	6.57**	8.66**
Bilrubin-UDPGT	0.988	0.849	1.229*	1.283**	1.282**	1.425**

Dose-levels (ppm)	Females					
	0	**50**	**110**	**250**	**560**	**1250**
Nmol/min/mg protein						
Recovery phase						
Cytochrome P450 nmol/mg protein	0.37					0.40
PROD Pmol/min/mg protein	13.25					12.28
BQ Nmol/min/mg protein	4.88					4.79
Bilrubin-UDPGT Nmol/min/mg protein	1.256					1.087

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

Gene transcription analysis (qPCR) revealed an increase in the transcription of genes which are induced by the activation of the CAR and/or PXR receptors (Cyp1a2, Cyp3a11, Ugt2b5 and Ugt1a1); no increase in the transcription of these particular genes was seen in the recovery phase treated animals. Cyp1a1 was not induced indicating that the Ah receptor has not been activated by isoflucypram administration (see table B.6.8-21 below).

Table B.6.8-21: Gene transcription (liver and pituitary) in female mice administered isoflucypram for 7 days

Dose-levels (ppm)	Females					
	0	**50**	**110**	**250**	**560**	**1250**
Treatment phase						
Cyp1a1	1.327	1.233	1.291	1.190	1.309	1.436
Cyp1a2	1.037	1.091	1.146	1.032	1.233*	1.372**
Cyp2b9	0.913	0.957	0.942	0.872	0.909	0.940
Cyp2b10	1.239	2.123**	3.350**	5.852**	11.292**	22.691**
Ugt2b5	0.927	1.069	1.197**	1.165**	1.437**	1.411**
Ugt1a1	0.959	1.044	1.031	1.074	1.220**	1.533**
Recovery phase						
Cyp1a1	0.985					1.007
Cyp1a2	0.721					0.775
Cyp2b9	0.706					0.679
Cyp2b10	1.013					1.169
Ugt2b5	0.949					0.951
Ugt1a1	0.881					0.903

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

Discussion and conclusion

Dietary administration of isoflucypram to female Wistar rats for 7 days, resulted in increased liver weights and enlarged liver (but no microscopic findings) from approximately 250 ppm (44 mg/kg bw/d) up to the top dose of 1250 ppm (224 mg/kg bw/d). There was no cell proliferation in the thyroid. Cell proliferation in the liver was observed at 250 ppm, but without a dose-response, it is unlikely this effect was induced by treatment with isoflucypram. An increase in total P450 was observed from the lowest dose tested of 50 ppm (8.9 mg/kg bw/d), with the majority of the increase being comprised of PROD. An increase in the phase II enzyme bili-UDGPT was also noted from 50 ppm. With regard to gene transcription, those genes involved in the activation of CAR and PXR receptors were found to be increased. Therefore under the conditions of this study isoflucypram is likely to operate via a CAR and/or PXR activation mode of action.

([redacted] *2018c)*

Discussion and conclusion on mechanistic studies for liver and thyroid effects

A series of dietary studies have been conducted to investigate the potential mechanism for the effects seen in the liver of rats and mice and the thyroid of rats. These comprised one 7-day and two 28-day studies in rats and a 7-day study in mice. All studies were conducted in females and the applicant has reasoned that this is the most

appropriate sex, because the liver effects observed in this sex were more severe; however, although this is true, the RMS considers that testing in males (or both sexes) would have been more appropriate, owing to the thyroid effects being observed in males or both sexes. The applicant has proposed that isoflucypram is an activator of CAR and/or PXR and the results of this set of studies appear to support this finding, based upon investigations conducted on several parameters including liver/thyroid weights, thyroid hormones, liver/thyroid histopathology, liver/thyroid cell proliferation, liver enzyme induction and gene transcription.

Consistent with the findings of the previously conducted repeated-dose studies, relative and/or absolute liver weights were increased after 7-days in rats and mice and to a greater extent after 28-days' administration in rats. Isoflucypram did not have any effect on thyroid weights (measured in rats only). The increase in liver weight was accompanied by an enlarged liver in rats at 28-days and in mice at 7 days. Where the tissues were examined microscopically, hepatocellular and thyroid follicular cell hypertrophy was found to be present in rats after 28-days (but not 7 days); no hypertrophy was observed in mice. Cell proliferation was measured after 7- and 28-days' exposure in rats and was found to be increased in the liver and thyroid (only the proliferation in the liver persisted into recovery). No conclusion could be drawn on the extent of cell proliferation in mice owing to the lack of a clear dose-response.

Analysis of liver enzyme induction and associated gene transcription indicated that a PXR and/or a CAR MoA is the most likely MoA. In both 28-day rat studies, BROD (associated with PXR activation) was increased as well as the genes associated with both BROD and PROD (associated with CAR activation). In the 7-day studies (in rats and mice) an increase in PROD and associated genes was more prominent.

Therefore it is likely that the effects observed in the liver and thyroid are a result of liver enzyme induction, arising from the activation of CAR and/or PXR.

B.6.8.3. Studies on endocrine disruption

Estrogenic and androgenic modalities

The androgenic/anti-andrognic activity (and effect on puberty) and the oestrogenic/anti-oestrogenic activity of isoflucypram have been investigated in a Hershberger and a Uterotrophic assay respectively.

Hershberger assay

The primary objective of this study was to investigate in a short-term assay (a modified weanling rat Hershberger assay), the androgenic or anti-androgenic potential of isoflucypram. This was achieved by assessing potential weight changes in the androgen-dependent sex accessory tissues following oral exposure for 10 days to the test substance alone or to a combination of the androgen testosterone propionate and isoflucypram. The second objective of this study was to determine whether isoflucypram has the potential to affect puberty by monitoring preputial separation during oral exposure for 30 days.

Study	BCS-CN88460 – Evaluation in the weanling rat Hershberger assay coupled with preputial separation assessment
Reference	[redacted]2012
Date performed	May-June 2012
Test facility	
Report reference	SA 11334
Guideline(s)	None
Deviations from the guideline	N/A
GLP	No
Test material	Isoflucypram ; batch NLL 8674-19-4; purity 98.6%
Study acceptable	Yes

Methods

Isoflucypram (in 0.5% aqueous mehylcellulose 400) was administered by gavage to 6/group immature male Sprague-Dawley rats; the study report does not make reference to any particular guideline but broadly follows OECD guidance document 115 (screening assay or use of weanling rats rather than the castrated adult rats as described in OECD TG 441). To assess the androgenic/anti androgenic properties of isoflucypram the substance

was administered for 10 days. Additionally, to investigate the potential of isoflucypram to affect puberty in male rats, preputial separation was monitored after administration of the test substance for 30 days. A fully validated method of analysis for isoflucypram in the gavage solution at the different concentrations tested is available (see document CA_B5 for further details).

To screen for androgenic properties the animals (6 weanlings /group) received a daily oral gavage dose of 800 mg/kg bw/d isoflucypram or vehicle control for 10 days. To screen for anti-androgenic activity the test subjects received a daily dose of testosterone propionate (subcutaneous injection) in addition to a daily oral gavage dose of vehicle control, or isoflucypram at 400 or 800 mg/kg bw/d. On the day of necropsy (study day 11, PND 33), preputial separation was recorded and any changes to the weights of the sex accessory tissues (epididymides, ventral prostate, seminal vesicle, Cowper's glands, and levator ani / bulbocavernosus muscles) were determined.

For the assessment of puberty, three additional groups of 6 immature male rats were dosed by daily oral gavage with vehicle control, 400 or 800 mg/kg bw/d for 30 days. The animals were then monitored daily from post-natal day 33 (PND 33), corresponding to study day 11, for preputial separation. The weights of the sex accessory tissues (epididymides, ventral prostate, seminal vesicle, Cowper's glands, and levator ani / bulbocavernosus muscles), testes, and liver were recorded at necropsy.

Results

Mortality, clinical signs and body weights

There were three deaths (one at 400 mg/kg bw/d and two at 800 mg.kg bw/d); one death was attributed to a gavage error and for the remaining two a cause of death could not be determined; neither of these deaths was considered to be related to treatment with isoflucypram. The only clinical sign observed was an increase in salivation at 400 and 800 mg/kg bw/d. In the androgenic activity group (10 days) body weights were reduced throughout the study and both body weight and body-weight-gain were lower than controls by the end of the study. There was no effect on body weight and body weight gain in the anti-androgenic groups (10 days) and body-weights for the 30-day puberty evaluation groups were only slightly lower than controls.

Table B.6.8-22: Body weight development in male rats administered isoflucypram for 10 days.

Dose-levels (ppm)	Body weight, g		Cumulative body weight gain, g	
	0	800	0	800
1	61.10	59.83		
2	66.35	61.82	5.25	1.98***
3	72.25	64.92*	11.15	5.08***
4	77.68	72.28	16.58	12.45**
5	83.47	76.52	23.37	16.68***
6	92.08	82.26*	30.98	23.86**
7	98.42	88.24*	37.32	29.84**
8	106.12	94.84*	45.02	36.44**
9	112.78	100.76*	51.68	42.36**
10	120.87	108.16*	59.77	49.76*

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Table B.6.8-23: Body weight and cumulative body weight gain in immature male rats administered isoflucypram by oral gavage for 30 days

	Isoflucypram, dose in mg/kg bw/day					
	Body weight, g			Cumulative body weight gain, g		
Day	0	400	800	0	400	800
1	60.80	60.32	61.07			
7	100.63	92.45	93.17	39.83	32.85*	31.38**
14	162.53	153.14	152.52	101.73	92.94	91.45
21	229.15	214.44	212.52	168.35	154.24	151.45
30	314.73	288.20	290.30	253.93	228.00	229.23

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Preputial seperation

In animals administered isoflucypram at 800 mg/kg bw/d on postnatal days 23 through 32, as expected, there was no preputial separation in either the control or the treated group. This indicates that isoflucypram did not act as an androgen in the immature male rat. In animals administered isoflucypram at 400 or 800 mg/kg bw/d with testosterone propionate at 1 mg/kg bw/d on postnatal days 23 through 32, as expected, preputial separation was noted in all animals of all groups. This indicates that isoflucypram did not act as an anti-androgen in the immature male rat treated with testosterone propionate.

There was no effect of oral gavage administration of isoflucypram at either 400 or 800 mg/kg bw/d for 30 days on the observation of preputial separation.

Table B.6.8-24: Age and body weight at the observation of partial and complete preputial separation in male rats administered isoflucypram by oral gavage for 30 days

		Isoflucypram, dose in mg/kg bw/day		
		0	**400**	**800**
Partial separation	Mean age, days	40.83 ± 1.33	42.80 ± 0.84	41.50 ± 1.76
	Mean weight, g	208.45 ± 20.27	212.30 ± 10.17	197.62 ± 10.92
Complete separation	Mean age, days	48.00 ± 2.45	49.00 ± 2.83	50.50 ± 3.08
	Mean weight, g	276.90 ± 33.32	264.22 ± 10.20	271.97 ± 7.80

Weight of androgen-sensitive and sex accessory organs

In animals tested for the androgenic effect of isoflucypram at 800 mg/kg bw/d, there was no effect of treatment on the weight of the five androgen-dependent sex accessory tissues after 10 days treatment. Terminal body weight was slightly decreased in the treated group, while both absolute and relative liver weight were increased compared to controls. Similarly, in animals administered isoflucypram at 400 or 800 mg/kg bw/d in conjunction with 1 mg/kg bw/d of testosterone propionate, to examine the anti-androgenic effect of isoflucypram, there was no effect on the weight of the five androgen-dependent sex accessory tissues. Terminal body weight was also unaffected in these animals.

Table B.6.8-25: Terminal body weight and weight of specific organs in immature male rats administered isoflucypram, either with or without testosterone propionate (TP), for 10 days

	Isoflucypram, dose in mg/kg bw/day				
	Isoflucypram alone		With testosterone propionate		
	0	**800**	**0 + TP**	**400 + TP**	**800 + TP**
Terminal body wt, g	130.88	118.74*	130.68	135.57	133.94
Liver, g	5.70	7.03**	5.59	7.16*	8.14**
Epididymides, mg	120.32	115.84	157.05	164.72	156.06
Ventral prostate, mg	67.80	62.52	118.38	130.20	121.44
Seminal vesicles, mg	40.65	40.34	227.25	232.40	240.08
Cowper's glands, mg	6.02	6.62	23.75	23.50	24.30
LABC, mg	136.65	124.20	275.13	283.52	284.80

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

In animals monitored for the onset of puberty after administration of isoflucypram at either 400 or 800 mg/kg bw/d for 30 days, there was a slight but not statistically significant decrease in terminal body weight and an increase in absolute and relative liver weight at 800 mg/kg bw/d, but no increase at either dose in the weight of either the testes or the androgen-dependent sex accessory tissues.

Table B.6.8-26: Terminal body weight and weight of specific organs in male rats administered isoflucypram for 30 days

	Isoflucypram, dose in mg/kg bw/day		
	0	**400**	**800**
Terminal body wt, g	324.67	298.82	297.72
Liver wt, g	14.76	15.37	16.19
Testis, g	2.715	7.684	2.601
Epididymides, mg	472.23	454.66	429.25
Ventral prostate, mg	292.52	231.14*	236.75*
Seminal vesicles, mg	666.37	509.54	552.25
Cowper's glands, mg	45.75	43.54	41.37

	Isoflucypram, dose in mg/kg bw/day		
	0	**400**	**800**
LABC, mg	706.62	577.64	619.23

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Conclusion

In a modified Hershberger assay in immature male rats, the oral gavage administration of isoflucypram for 10 days, either with or without testosterone propionate, at either 400 or 800 mg/kg bw/d, did not affect either preputial separation or the weight of androgen-sensitive organs or sex accessory tissues. Additionally, the administration of isoflucypram for 30 days at either 400 or 800 mg/kg bw/d had no effect on the timing of preputial separation or the weight of androgen-sensitive and sex-accessory organs. Based on these results, isoflucypram does not have either an androgenic or anti-androgenic activity in this assay *in vivo*.

([redacted] 2012)

Uterotrophic assay in immature animals

The objectives of this study were to investigate in the immature rat uterotrophic assay the potential of isoflucypram to interfere with uterine growth or to modify the timing of vaginal opening.

Study	BCS-CN88460 – Evaluation in the immature rat uterotrophic assay coupled with vaginal opening
Reference	[redacted] 2011
Date performed	Dec 2019 – Jan 2011
Test facility	[redacted]
Report reference	SA 10453
Guideline(s)	None, but similar to OECD 440
Deviations from the guideline	N/A
GLP	No
Test material	Isoflucypram; batch LJGD563-1-2; purity 93.6%
Study acceptable	Yes

Methods

Groups of 6 immature female Sprague Dawley rats (19 days old) were dosed daily by oral gavage for 3 days with either the vehicle (0.5% aqueous mehylcellulose 400) or isoflucypram at 400 or 800 mg/kg bw/d. At 24 hours after the end of the dosing period, vaginal opening was assessed and uterine weight was recorded. A separate group was administered estradiol benzoate (EB) as a positive control for the induction of an uterotrophic response.

To identify pubertal effects, additional groups of 6 immature female Sprague Dawley rats (19 days old) were dosed on a daily basis by oral gavage for 20 days with either the vehicle (0.5% aqueous mehylcellulose 400) or with isoflucypram at 400 or 800 mg/kg bw/d. The day of vaginal opening was recorded from day 10 onwards. The uterine and liver weights were recorded 24 hours after the end of the dosing period. A validated method of analysis for isoflucypram in the gavage solution at the different concentrations tested is not available (see document CA_B5 for further details). However, as this study is a mechanistic sudy relevant to the endocrine disruption hazard identification potential of isoflucypram rather than to the characterisation of its dose-response, the study is considered to be acceptable.

Results

3-day treatment

At 800 mg/kg bw/d, the maximum tolerated dose was exceeded, as mortality, severe clinical signs, and marked effects on body weight parameters were observed. Specifically, one animal in this group was sacrificed prematurely on study day 3 due to severe clinical signs (piloerection, reduced motor activity, prostration, and wasted appearance) and a body weight loss of 7 g. In animals surviving to terminal sacrifice, one case of reduced motor activity was noted on study day 3. Mean body weight was lower than the controls by 18% on study day 2 and by 23% on study day 3. Overall, there was a mean cumulative body weight loss of 1.3 g compared to a gain of 8 g in the controls.

At 400 mg/kg bw/d, there were neither mortalities, nor clinical signs observed. Mean body weight was lower than the controls by 11% on study day 2 and by 9% on study day 3. Overall mean cumulative body weight gain between study days 1 and 3 was reduced by 42% compared to the controls.

Table B.6.8-27: Body weight and cumulative body weight gain in female rats administered isoflucypram or estradiol benzoate for 3 days

Day	Isoflucypram, dose in mg/kg bw/day			Estradiol benzoate
	0	400	800	0.08 mg/kg bw/day
1	39.8	39.1	38.1	39.8
2	43.5	38.7	35.7*	42.1
3	47.8	43.7	36.8**	47.2
Gain, days 1-3, g	8.0	4.6	-1.3**	7.5

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

At 800 mg/kg bw/d, vaginal opening on study day 4 was not examined because the maximum tolerated dose was exceeded. At 400 mg/kg bw/d, as expected, no vaginal opening was noted prior to necropsy on study day 4 in these immature animals (as in controls).

At necropsy, mean terminal body weight was 19% and 9% lower than in controls at 800 and 400 mg/kg bw/d respectively. Mean uterus weights were not affected by the treatment at either dose.

Table B.6.8-28: Terminal body weight and uterine weight in female rats administered either isoflucypram or estradiol benzoate for 3 days

		Isoflucypram, mg/kg bw/day			Estradiol benzoate
		0	400	800	0.08 mg/kg bw/day
Terminal body wt, g		53.4	48.7	43.0**	50.7
Wet uterine weight	Absolute, mg	0.0263	0.0267	0.0260	0.0540**
	% body wt	0.04918	0.05494	0.06146	0.10825*
Blotted uterine wt	Absolute, mg	0.0244	0.0242	0.0233	0.0419*
	% body wt	0.04563	0.04979	0.05511	0.08474*

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

20-day treatment

At 800 mg/kg bw/d, the maximum tolerated dose was exceeded as mortality, severe clinical signs, and marked effects on body weight parameters were observed. One animal in this group was found dead on study day 3 after showing severe clinical signs (piloerection, reduced motor activity, uncoordinated movements) and a weight loss of 5.7 g prior to death. A second animal was sacrificed prematurely on study day 4 due to severe clinical signs (piloerection, general pallor, reduced motor activity, hunched posture, and wasted appearance). A body weight loss of 7.7 g was noted for this animal on study day 4. In animals surviving to terminal sacrifice, one case of reduced motor activity and wasted appearance was noted on study day 3. Mean body weight was lower than the controls by between 6 and 22% throughout the study period, the effect being most pronounced on study days 3 and 4. Overall mean cumulative body weight gain between study days 1 and 30 was reduced by 10% compared to the controls.

At 400 mg/kg bw/day, there were neither mortalities, nor treatment-related clinical signs of toxicity throughout the study. Mean body weight was transiently lower than the controls by 7% on study days 2 and 3, and was similar to the controls at the end of the treatment period. Overall mean cumulative body weigh gain between study days 1 and 20 was similar to the controls.

Table B.6.8-29: Body weight and cumulative body weight gain in female rats administered isoflucypram for 20 days

	Isoflucypram, dose in mgkg bw/day		
Day	0	400	800
1	40.0	39.7	39.0
2	42.9	39.9	37.0
3	48.5	44.9	37.9**
4	52.3	49.7	40.9
5	57.3	54.1	50.0
6	61.9	59.4	54.3

	Isoflucypram, dose in mgkg bw/day		
Day	**0**	**400**	**800**
7	67.2	63.8	57.4
14	107.3	102.8	94.4
20	145.9	149.1	135.4
Gain, days 1-20, g	105.8	109.4	95.3

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

At 800 mg/kg bw/d, mean age and mean body weight at vaginal opening were not recorded since the maximum tolerated dose was exceeded. At 400 mg/kg bw/d, there were no treatment-related effects on the mean age or mean weight at vaginal opening.

Table B.6.8-30: Mean age and body weight at vaginal opening in female rats administered isoflucypram for 20 days

	Isoflucypram, dose in mg/kg bw/day	
	0	**400**
Mean age, days	33.17	33.50
Mean body weight, g	115.17	111.22

At necropsy, mean terminal body weight was 7% lower than in controls at 800 mg/kg bw/d. There were no effects on terminal body weight at 400 mg/kg bw/d. Mean liver weights were significantly increased by 21-30% and 24-27% compared to controls at 800 and 400 mg/kg bw/d respectively. Mean uterine weights were not affected by treatment at either dose.

Table B.6.8-31: Terminal body weight and uterine weight in female rats administered isoflucypram for 20 days

		Isoflucypram, dose in mg/kg bw/day		
		0	**400**	**800**
Terminal body wt, g		150.7	153.2	140.6
Liver weight	Absolute, g	6.99	8.49*	8.12
	% body wt	4.440	5.521**	5.755**
Wet uterine weight	Absolute, mg	0.2580	0.3049	0.1933
	% body wt	0.17074	0.20154	0.13950
Blotted uterine weight	Absolute, mg	0.2453	0.2545	0.1866
	% body wt	0.16234	0.16746	0.13466

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Conclusion

In this modified uterotrophic assay in immature rats, the oral gavage administration of isoflucypram to females for either 3 or 20 days did not alter either vaginal opening (only determined at 400 mg/kg bw/d due to exceedance of the MTD at 800 mg/kg bw/d), nor was there any effect of treatment on either wet or blotted uterine weight up to the top dose of 800 mg/kg bw/d. Based on these results, isoflucypram does not have an estrogenic effect in this assay *in vivo*.

(*2011)*

Thyroid modality

Mild effects on the thyroid gland (increased weight and/or follicular cell hypertrophy and/or colloid alteration and/or pigmentation) were seen in rats in the short-term, carcinogencicity and 2-generation reproductive toxicity studies. There were no effects on the thyroid in mice or dogs.

Mechanistic investigations (see section B.6.8.2) have shown that these changes were associated with increases in TSH and that the most likely MoA (mode of action) underpinning the induction of these effects is activation of CAR/PXR in the liver followed by enzyme induction, including T4-UDPGT, increased clearance of T4, stimulation of the pituitary with increased secretion of TSH leading to stimulation of the thyroid and follicular cell hypertrophy.

It is well established that there are large quantitative differences between rats and humans in the regulation of thyroid homeostasis (Boobis et al., 2006; Lewandowski et al., 2004; Capen, 1997; Capen, 1998; Colnot and Dekant, 2017). Therefore, the thyroid effects seen in the rat as a secondary consequence of liver enzyme induction are

considered to be not relevant to humans by the RMS, especially when taking into account the mild nature and low incidence of the effects observed.

Conclusion on endocrine disruption (ED)

An assessment for potential endocrine disrupting properties of isoflucypram in line with the new EFSA/ECHA guidance (https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2018.5311) and the recently published scientific criteria (Regulation 605/2018) has been provided by the applicant. This is reproduced in full in Appendix 1 to this B6 document. The RMS has evaluated the assessment. The following summary conclusions are reached by the RMS.

Estrogenity (E), androgencity (A) and steroidogenesis (S) modalities

No effects on reproduction or reproductive organs were observed in a modern 2-generation study in rats, pre-natal developmental toxicity studies in rats and rabbits and repeated dose toxicity studies in rats, mice and dogs. The 2-generation study included investigations of all the required ED-sensitive parameters (with the exception of nipple retention). In addition, no (anti-)androgenic or oestrogenic activity was seen in a modified Hershberger assay and uterotrophic test in immature animals. Overall, it can be concluded that isoflucypram is not an ED in relation to these modalities.

Thyroid (T) modality

Endocrine-mediated thyroid toxicity was seen in the rat. Based upon the large quantitative differences between rats and humans in the regulation of thyroid homeostasis, these effects are considered to be not relevant to humans, especially when taking into account the mild nature and low incidence of the observed changes. Overall, it can be concluded that isoflucypram is not a thyroid ED in humans.

Overall

Isoflucypram is not an ED of potential relevance to human health in accordance with the recently published scientific criteria (Reg 605/2018).

References

Boobis AR, Cohen SM, Dellarco V, McGregor D, Meek ME, Vickers C, Willcocks D and Farland W, 2006. IPCS framework for analyzing the relevance of a cancer mode of action for humans. Crit Rev Toxicol, 2006;36(10):781-92.

Capen CC (1997) Mechanistic data and risk assessment of selected toxic end points of the thyroid gland. Toxicol Pathol. ;25(1):39-48.

Capen CC (1998). Correlation of mechanistic data and histopathology in the evaluation of selected toxic endpoints of the endocrine system. Toxicol. Lett.;102-103, 405-409.

Colnot T, Dekant W. (2017) Approaches for grouping of pesticides into cumulative assessment groups for risk assessment of pesticide residues in food. Regul Toxicol Pharmacol.;83:89-99. doi: 10.1016/j.yrtph.2016.12.004.

Lewandowski TA, Seeley MR, Beck BD. (2004) Interspecies differences in susceptibility to perturbation of thyroid homeostasis: a case study with perchlorate. Regul Toxicol Pharmacol.;39(3):348-62.

B.6.8.4. Immunotoxicity

No specific immunotoxicity study with isoflucypram is available. However, an assessment of the immunotoxicity potential of isoflucypram can be performed by considering the available repeat dose toxicity, carcinogencicity and reproductive toxicity studies.

Various repeat dose toxicity studies conducted with isoflucypram in the rat, mouse, and dog show that there is no indication of an immunotoxic effect in adult animals. The weight of the spleen, thymus and lymph nodes were unaffected in all three species, and there was no alteration in morphology in these organs which would suggest an effect of isoflucypram on the immune system. In addition, there were no relevant effects on any haemathological parameters.

There were also no relevant or specifc effects on spleen and thymus in rat and rabbit foetuses and in rat pups in the available reproductive toxicity studies.

It can therefore be concluded that isoflucypram does not affect the immune system, and an *in vivo* immunotoxicity study is not required.

B.6.9. MEDICAL DATA AND INFORMATION

B.6.9.1. Medical surveillance on manufacturing plant personnel and monitoring studies

No medical problems related to handling of isoflucypram during the piloting phase have been reported to the plant Medical Department and HSE Management. There is no production currently ongoing. All plant employees undergo annual medical examinations, including clinical examinations, clinical-pathology, spirometry, audiogram and ECG (Steffens, 2017).

B.6.9.2. Data collected on humans

There are no publications on human poisoning cases.

B.6.9.3. Direct observation

No poisoning cases have come to the attention of Bayer AG, CropScience Division.

B.6.9.4. Epidemiological studies

There are no published epidemiological studies.

B.6.9.5. Diagnosis of poisoning (determination of active substance, metabolites), specific signs of poisoning, clinical test

No human cases have been reported. In acute toxicity studies in animals, no specific signs of exposure have been seen.

B.6.9.6. Proposed treatment: first aid measures, antidotes, medical treatment

First aid:

Remove patient from exposure / terminate exposure

- Thorough skin decontamination with copious amounts of water and soap, if available with polyethylene glycol 300 followed by water.

  Note: most formulations with this active ingredient can be decontaminated with water (and soap), so for formulations polyethylene glycol 300 is not required.

- Flushing of the eyes with lukewarm water for 15 minutes.

- Induction of vomiting does not seem to be required. It should only be considered if a very large amount has been swallowed, if the ingestion was less than one hour ago, and if the patient is fully conscious.

  Induced vomiting can remove maximum 50% of the ingested substance.

  Note: Induction of vomiting is forbidden if a formulation containing organic solvents has been ingested!

Treatment:

- Gastric lavage does not seem to be required in regard of the low toxicity.

- The application of activated charcoal and sodium sulphate (or other cathartic) may be considered in significant ingestions.

- As there is no antidote, treatment has to be symptomatic and supportive.

B.6.10. REFERENCES RELIED ON

LITERATURE SEARCH

A literature review was carried out (by the Applicant) for isoflucypram and its metabolites according to the requirements of Article 8(5) of Regulation (EC) No 1107/2009. The review itself was performed in accordance with the EFSA Guidance document as published in EFSA Journal 2011; 9(2):2092.

The literature search covered the period from January 2008 to October 2017. A broad range of databases (Agricola, Biosis, CABA, Chemical Abstracts, DRUGU, EMBASE, Esbiobase, IPA, Medline, PQSciTech, Scisearch, Toxcenter, FSTA) was searched. The search terms included the IUPAC name, CAS name/number, common names, codes and abbreviations, molecular structure, molecular formula, molar mass and other names/codes of the active substance and its main metabolites.

No publications were identified by the search. Therefore, a subsequent assessment of relevance and reliability was not required.

Data Point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
KCA 5.1.1 / 01	[redacted]	2017	Amendment no 1 to final report - [Pyrazole-4-14C]BCS-CN88460 - Absorption, distribution, excretion and metabolism in the rat [redacted] Bayer Report No.: EnSa-16-1015 Date: 2017-09-21 GLP/GEP: Yes, unpublished	Yes	Yes	New data for a new active substance	Bayer	No
KCA 5.1.1 / 02	[redacted]	2017	Amendment no 1: [Phenyl-UL-14C]BCS-CN88460 - Absorption, distribution, excretion and metabolism in the rat [redacted] Bayer Report No.: EnSa-16-1014 Date: 2017-09-26 GLP/GEP: Yes, unpublished	Yes	Yes	New data for a new active substance	Bayer	No
KCA 5.1.1 / 03	[redacted]	2017	[Pyrazole-4-14C]BCS-CN88460: Distribution of the total radioactivity in male and female rats determined by quantitative whole body autoradiography, determination of the exhaled 14CO2, and pilot metabolism experiments	Yes	Yes	New data for a new active substance	Bayer	No

			[redacted] Bayer Report No.: EnSa-16-1012 Date: 2017-09-21 GLP/GEP: Yes, unpublished					
KCA 5.1.1 / 04	[redacted]	2017	Amendment 1 to [phenyl-UL-14C]BCS-CN88460: Tissue distribution and excretion of radioactivity in the rat by quantitative whole body autoradiography - Amended final report 1 [redacted] Bayer Report No.: EnSa-16-1023 Date: 2017-02-03 GLP/GEP: Yes, unpublished	Yes	Yes	New data for a new active substance	Bayer	No
KCA 5.1.1 / 05	[redacted]	2017	Amendment no. 1: [Pyrazolyl-4-14C]BCS-CY26497: Pilot metabolism experiments in male rats [redacted] Bayer Report No.: EnSa-16-1013 Date: 2017-09-28 GLP/GEP: Yes, unpublished	Yes	Yes	New data for a new active substance	Bayer	No
KCA 5.1.2 / 01	Lagojda, A.; Doebbe, A.	2017	[Pyrazole-4-14C]BCS-CN88460 - Metabolic stability and profiling in liver microsomes from different animals and humans for inter-species comparison Bayer AG, Crop Science Division, Monheim, Germany Bayer Report No.: EnSa-17-0305 Date: 2017-08-24 GLP/GEP: Yes, unpublished	No	Yes	New data for a new active substance	Bayer	No
KCA 5.2.1 / 01	[redacted]	2014	BCS-CN88460 technical - Acute oral toxicity study in the rat (up and down procedure) [redacted] Bayer Report No.: 14/069-001P	Yes	Yes	New data for a new active substance	Bayer	No

			Date: 2014-05-12 GLP/GEP: Yes, unpublished					
KCA 5.2.2 / 01	[redacted]	2014	BCS-CN88460 technical - Acute dermal toxicity study in rats [redacted] Bayer Report No.: 14/069-002P Date: 2014-04-29 GLP/GEP: Yes, unpublished	Yes	Yes	New data for a new active substance	Bayer	No
KCA 5.2.3 / 01	[redacted]	2014	BCS-CN88460 technical - Acute inhalation toxicity study (nose-only) in the rat [redacted] Bayer Report No.: 14/069-004P Date: 2014-11-17 GLP/GEP: Yes, unpublished	Yes	Yes	New data for a new active substance	Bayer	No
KCA 5.2.4 / 01	[redacted]	2014	BCS-CN88460 technical - Acute skin irritation study in rabbits [redacted] Bayer Report No.: 14/069-006N Date: 2014-04-18 GLP/GEP: Yes, unpublished	Yes	Yes	New data for a new active substance	Bayer	No
KCA 5.2.5 / 01	[redacted]	2014	BCS-CN88460 technical - Acute eye irritation study in rabbits [redacted] Bayer Report No.: 14/069-005N Date: 2014-07-29 GLP/GEP: Yes, unpublished	Yes	Yes	New data for a new active substance	Bayer	No
KCA 5.2.5 / 02	Váliczkó, É.	2014	BCS-CN88460 technical - In vitro eye irritation test in isolated chicken eyes CiToxLAB Hungary Ltd., Veszprém, Szabadságpuszta, Hungary Bayer Report No.: 14/069-038CS Date: 2014-05-13 GLP/GEP: Yes, unpublished	Yes	Yes	Data requirement of Regulation EC 1107/2009	Bayer	No

KCA 5.2.6 / 01	[redacted]	2015	BCS-CN88460 technical - Local lymph node assay in the mouse [redacted] Bayer Report No.: 14/069-037E Date: 2015-06-05 GLP/GEP: Yes, unpublished	Yes	Yes	Data requirement of Regulation EC 1107/2009	Bayer	No
KCA 5.2.7 / 01	Spohr, C.	2018	Isoflucypram technical: Cytotoxicity assay in vitro with BALB/c 3T3 Cells: Neutral red (NR) test during simultaneous irradiation with artificial sunlight Envigo CRS GmbH, Rossdorf, Germany Bayer Report No.: 1858400 Date: 2018-02-05 GLP/GEP: Yes, unpublished	No	Yes	Data requirement of Regulation EC 1107/2009	Bayer	No
KCA 5.3.1 / 01	[redacted]	2017	BCS-CN88460 - Exploratory 28-day toxicity study in the rat by dietary administration [redacted] Bayer Date: 2013-09-04 GLP/GEP: No, unpublished	Yes	No	Mechanistic study supporting hazard assessment	Bayer	No
KCA 5.3.1 / 02	[redacted]	2012	BCS-CN88460 - Preliminary 28-day toxicity study in the mouse by dietary administration [redacted] Bayer Report No.: SA 11309 Date: 2012-11-29 GLP/GEP: No, unpublished	Yes	No	Mechanistic study supporting hazard assessment	Bayer	No
KCA 5.3.1 / 03	[redacted]	2014	BCS-CN88460 - Preliminary 28-day toxicity study in the dog by dietary administration [redacted] Bayer Report No.: SA 12107 Date: 2014-11-27 GLP/GEP: No, unpublished	Yes	No	Mechanistic study supporting hazard assessment	Bayer	No
KCA 5.3.1 / 04	[redacted]	2011	Reference compounds for hepatotoxicity exploratory	Yes	No	-	Bayer	No

			28-day toxicity study in the rat by gavage [redacted] Bayer Report No.: SA 06181 MRID#: 48387101 Date: 2007-11-16 GLP/GEP: No, unpublished					
KCA 5.3.2 / 01	[redacted]	2017	BCS-CN88460 - 90-day toxicity study in the rat by dietary administration - Final report amendment no. 1 [redacted] Bayer Date: 2014-05-21 GLP/GEP: Yes, unpublished	Yes	Yes	New data for a new active substance	Bayer	No
KCA 5.3.2 / 02	[redacted]	2013	BCS-CN88460 - 90-day toxicity study in the mouse by dietary administration [redacted] Bayer Date: 2013-12-12 GLP/GEP: Yes, unpublished	Yes	Yes	New data for a new active substance	Bayer	No
KCA 5.3.2 / 03	[redacted]	2015	BCS-CN88460: 90-Day toxicity study in the dog by dietary administration [redacted] Bayer Report No.: SA 13272 Date: 2015-05-05 GLP/GEP: Yes, unpublished	Yes	Yes	New data for a new active substance	Bayer	No
KCA 5.3.2 / 04	[redacted]	2017	BCS-CN88460 - Chronic toxicity study in the dog by dietary administration [redacted] Bayer Report No.: SA 14092 Date: 2017-09-19 GLP/GEP: Yes, unpublished	Yes	Yes	New data for a new active substance	Bayer	No
KCA 5.4.1 / 01	Sokolowski, A.	2014	BCS-CN88460; technical: Salmonella typhimurium reverse mutation assay Harlan Cytotest Cell Research GmbH (Harlan CCR), Rossdorf, Germany	No	Yes	New data for a new active substance	Bayer	No

			Bayer Report No.: 1614801 Date: 2014-05-19 GLP/GEP: Yes, unpublished					
KCA 5.4.1 / 02	Bohnenberger, S.	2014	BCS-CN88460, technical: Chromosome aberration test in human Lymphocytes in vitro Harlan Cytotest Cell Research GmbH (Harlan CCR), Rossdorf, Germany Bayer Report No.: 1614803 Date: 2014-07-23 GLP/GEP: Yes, unpublished	No	Yes	New data for a new active substance	Bayer	No
KCA 5.4.1 / 03	Wollny, H. E.	2014	BCS-CN88460, technical: Gene mutation assay in Chinese hamster V79 cells in vitro (V79 / HPRT) Harlan Cytotest Cell Research GmbH (Harlan CCR), Rossdorf, Germany Bayer Report No.: 1614802 Date: 2014-06-04 GLP/GEP: Yes, unpublished	No	Yes	New data for a new active substance	Bayer	No
KCA 5.4.2 / 01	[redacted]	2014	BCS-CN88460, technical - Micronucleus assay in bone marrow cells of the mouse [redacted] Bayer Report No.: 1614804 Date: 2014-05-07 GLP/GEP: Yes, unpublished	Yes	Yes	New data for a new active substance	Bayer	No
KCA 5.5 / 01	[redacted]	2018	BCS-CN88460: Chronic toxicity and carcinogenicity study in the wistar rat by dietary administration [redacted] Bayer Report No.: SA 13266 Date: 2018-01-26 GLP/GEP: Yes, unpublished	Yes	Yes	New data for a new active substance	Bayer	No
KCA 5.5 / 02	[redacted]	2017	BCS-CN88460 - Carcinogenicity study in the c57bl/6j mouse by dietary administration [redacted]	Yes	Yes	New data for a new active substance	Bayer	No

			Bayer Report No.: SA 13273 Date: 2017-06-27 GLP/GEP: Yes, unpublished					
KCA 5.6.1 / 01	[redacted]	2018	BCS-CN88460 technical: Two generation reproductive performance study by dietary administration to han wistar rats [redacted] Bayer Date: 2016-06-29 GLP/GEP: Yes, unpublished	Yes	Yes	New data for a new active substance	Bayer	No
KCA 5.6.2 / 01	[redacted]	2017	BCS-CN88460 - Developmental toxicity study in the rat by gavage [redacted] Bayer Report No.: SA 14192 Date: 2017-09-29 GLP/GEP: Yes, unpublished	Yes	Yes	New data for a new active substance	Bayer	No
KCA 5.6.2 / 02	[redacted]	2017	BCS-CN88460 - Developmental toxicity study in the rabbit by gavage - Final report [redacted] Bayer Report No.: SA 15122 Date: 2017-05-15 GLP/GEP: Yes, unpublished	Yes	Yes	New data for a new active substance	Bayer	No
KCA 5.7.1 / 01	[redacted]	2017	BCS-CN88460 - An acute neurotoxicity study in the rat by oral administration [redacted] Bayer Report No.: SA 15004 Date: 2017-07-05 GLP/GEP: Yes, unpublished	Yes	Yes	New data for a new active substance	Bayer	No
KCA 5.8.1 / 01	[redacted]	2011	BCS-CN45153 - Exploratory 28-day toxicity study in the rat by dietary administration (summary report) [redacted] Bayer	Yes	No	To support toxicological relevance of impurity	Bayer	No

			Report No.: SA 10158 Date: 2011-06-29 GLP/GEP: No, unpublished					
KCA 5.8.1 / 02	[redacted]	2010	BCS-CN45153 - Preliminary 28-day toxicity study in the mouse by dietary administration [redacted] Bayer Report No.: SA 09400 Date: 2010-11-16 GLP/GEP: No, unpublished	Yes	No	To support toxicological relevance of impurity	Bayer	No
KCA 5.8.1 / 03	[redacted]	2011	BCS-CN45153 - Evaluation in the immature rat uterotrophic assay coupled with vaginal opening [redacted] Bayer Report No.: SA 10312 Date: 2011-03-10 GLP/GEP: No, unpublished	Yes	No	To support toxicological relevance of impurity	Bayer	No
KCA 5.8.2 / 01	[redacted]	2013	BCS-CN88460 - Mechanistic 28-day toxicity study in the female rat by dietary administration (hepatotoxicity and thyroid hormone investigations) [redacted] Bayer Report No.: SA 12190 Date: 2013-09-27 GLP/GEP: No, unpublished	Yes	No	Mechanistic study supporting hazard assessment	Bayer	No
KCA 5.8.2 / 02	[redacted]	2018	BCS-CN88460 - 7-day mechanistic toxicity study for liver and thyroid cell proliferation in female Wistar rats [redacted] Bayer Report No.: SA 15054 Date: 2018-02-22 GLP/GEP: No, unpublished	Yes	No	Mechanistic study supporting hazard assessment	Bayer	No
KCA 5.8.2 / 03	[redacted]	2018	BCS-CN88460 - Mechanistic 7-day toxicity study for liver and thyroid cell proliferation in the C57BL/6J female mouse [redacted] Bayer	Yes	Yes	Mechanistic study supporting hazard assessment	Bayer	No

			Report No.: SA 14037 Date: 2018-02-14 GLP/GEP: Yes, unpublished					
KCA 5.8.2 / 04	[redacted]	2018	BCS-CN88460 - 28-day mechanistic toxicity study for liver and thyroid cell proliferation in female Wistar rats [redacted] Bayer Report No.: SA 15258 Date: 2018-02-22 GLP/GEP: No, unpublished	Yes	Yes	Mechanistic study supporting hazard assessment	Bayer	No
KCA 5.8.3 / 01	[redacted]	2012	BCS-CN88460 - Evaluation in the weanling rat Hershberger assay coupled with preputial separation assessment [redacted] Bayer Report No.: SA 11334 Date: 2012-12-07 GLP/GEP: No, unpublished	Yes	No	Mechanistic study supporting hazard assessment	Bayer	No
KCA 5.8.3 / 02	[redacted]	2011	BCS-CN88460 - Evaluation in the immature rat uterotrophic assay coupled with vaginal opening [redacted] Bayer Report No.: SA 10453 Date: 2011-05-05 GLP/GEP: No, unpublished	Yes	No	Mechanistic study supporting hazard assessment	Bayer	No
KCA 5.9 / 01	Steffens, W.	2017	Summary of medical data known for isoflucypram Bayer Date: 2017-09-26 GLP/GEP: n.a., unpublished	No	No	New data for a new active substance	Bayer	No

APPENDIX 1

Human health assessment of the endocrine disruption potential of isoflucypram provided by Bayer

1. Summary

This document analyzes the available toxicological data on the interactions of isoflucypram with the estrogen, androgen, steroidogenesis, and thyroid (E, A, S, and T) modalities, and examines whether or not isoflucypram has the potential to cause endocrine-mediated adverse effects in humans. These data were gathered, then the relevant studies were analyzed based on the new endocrine disruptor (ED) identification criteria (Commission Regulation (EU) 2018/605), and using the ECHA-EFSA guidance (referred to as the ED guidance) for the identification of endocrine disruptors in the criteria of Regulations (EU) No. 528/2012 and (EC) No 1107/2009. This assessment considers whether the data indicate that the EU ED criteria are met (Commission Regulation (EU) 2018/605).

A full dataset of regulatory studies was available, supplemented by relevant mechanistic information.
In studies with isoflucypram conducted in the rat, mouse, and dog, the liver was consistently identified as a target organ, with increased liver weight, altered morphology, and (where measured) induction of specific Phase I and Phase II enzymes. There was no reproductive or developmental effect observed, and long-term studies showed that isoflucypram is not oncogenic.

An assessment of data sufficiency conducted according to the ED guidance shows that E, A, and S-mediated parameters have been sufficiently investigated. Since no effects were observed on any of these parameters, it can be concluded that the ED criteria have not been met.

T-mediated parameters are also considered to have been sufficiently investigated, with effects observed only in the rat. Thyroid weight was increased at high doses, with increased TSH (thyroid stimulating hormone) observed in short-term studies, but the concentrations of T3 (triiodothyronine) and T4 (thyroxine) were not affected. There were very slight histological effects in the thyroid of rats at higher doses, indicating a slight increase in thyroid activity.

In a mode-of-action analysis, the guideline and mechanistic studies conducted in the rat have been assessed with regard to the effects of isoflucypram on thyroid hormone homeostasis and the possible mode of action responsible for the thyroid effects. It can be concluded that the isoflucypram-induced increase in thyroid weight and increase in TSH concentrations are due to a liver-mediated mode of action which is considered to be not relevant for humans.

Overall, based on the data available for isoflucypram, the ED criteria are not met.

2. Introduction

The new ED (endocrine disruptor) identification criteria (Commission Regulation (EU) 2018/605) that will be applicable to pesticides from November 10th, 2018 onwards, refer to the commonly admitted definitions of an ED (WHO/IPCS 2002) and of adverse effects (WHO/IPCS 2009). In line with the opinion of the European Food Safety Authority (EFSA 2013), the new ED criteria also clarify that an endocrine mode of action is not a (eco)toxicological hazard in itself.

The implementation of the new ED criteria relies on the ECHA-EFSA guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009, thereafter referred to as the ED Guidance. The pre-publication version (June 2018) of the ED Guidance was used in the present position paper.

According to the European scientific criteria for the determination of endocrine disrupting properties for plant protection products (Commission Regulation (EU) 2018/605), an active substance shall be considered as having endocrine disrupting properties that may cause adverse effects on non-target organisms if it is a substance that meets all of the following criteria:

(1) it shows an adverse effect in an intact organism or its progeny;
(2) it has an endocrine mode of action, *i.e*. it alters the function(s) of the endocrine system;
(3) the adverse effect is a consequence of the endocrine mode of action.

The European Commission also recommends an assessment of the available relevant scientific data based on a weight of evidence (WoE) approach considering, among others, (i) both positive and negative results, (ii) the quality and consistency of the data, considering the pattern and coherence of the results within and between studies of a similar design and across different species, and (iii) the biological plausibility of the link between the adverse effects and the endocrine mode of action (i.e. endocrine activity). Adverse effects that are non-specific secondary consequences of other toxic effects shall not be considered for the identification of the substance as an endocrine disruptor.

The WoE presented in this document synthesizes the available scientific data relevant for assessing potential endocrine-disrupting properties of isoflucypram in five Chapters. Data were interpreted in the light of recommendations from the ED Guidance (2018). Chapters 3 and 4 of the present document are dedicated to the assembly and assessment of lines of evidence for adverse effects and endocrine activity of isoflucypram (according to the ED Guidance chapter 3.3) in standard toxicity tests, also considering effects secondary to other toxicities. In Chapter 3 findings related to the EAS-modalities are evaluated. Chapter 4 considers all findings related to the T-modality. Subsequently data sufficiency is assessed (following the ED Guidance, an assessment of data sufficiency (according to the ED Guidance chapters 3.4.1 and 3.4.2)).

Chapter 4 also includes a detailed Mode of Action analysis combining the interpretation of the observed findings with the existing knowledge on the mode of action in order to evaluate the plausibility of biological links between the two.

Chapter 5 includes the overall conclusion based on this assessment.

3. Integrated lines of evidence for adverse effects and endocrine activity of isoflucypram (criterion 1 and 2) for the EAS modalities

3.1. Assessment of lines of evidence for the EAS modalities

Guideline and mechanistic studies conducted in the relevant laboratory species have been conducted and assessed with regard to the effects of isoflucypram on endocrine homeostasis. The key information from these studies has been extracted and will be provided in two phases, in summary format in this document and then later detailed data tables will be submitted. This summary considers the weight of the available evidence to assess the potential for endocrine activity and adversity to determine if the criteria are met for endocrine disruption.

Original data can be found in the study reports listed in Table 1 as submitted in support of the isoflucypram application for approval under Regulation (EU) No 1107/2009.

Table 1: Studies containing data addressing E, A, and S modalities.

Species	Duration / type	Reference
Rat	28 days	[redacted]2017;
	90 days	[redacted]2017;
	2 years	[redacted]2018;
	2-generation reproduction	[redacted]2018;
	Developmental toxicity	[redacted]2017;
	Immature female rat	[redacted]2011;
	Immature male rat	[redacted]2012;
Mouse	28 days	[redacted] 2012;
	90 days	[redacted]2013;
	18 months	[redacted] 2017;
Dog	28 days	[redacted]2014;
	90 days	[redacted] 2015;
	One year	[redacted]2017;
Rabbit	Developmental toxicity	[redacted] 2017;

3.1.1. Evidence for endocrine activity

The repeat-dose studies in mouse, rat, and dog ranging from 28 days through to one year (dog), 18 months (mouse), or two years (rat) require the assessment of weight and macro- and microscopic observations in a number of endocrine organs, as shown in Table 2. These studies are conducted in accordance with OECD test guidelines in effect at the time of the study.

Table 2: Endocrine-related organs which are weighed or examined at macro- or microscopic levels in repeat-dose studies in the rat (R), mouse (M), and dog (D).

	28-day		90-day		Long-term		2-gen repro	
Organ	**Weight**	**Microsc.**	**Weight**	**Microsc.**	**Weight**	**Microsc.**	**Weight**	**Microsc.**
Pancreas		R[1]M[1]		RMD		RMD		
Thymus	RM	RM	RMD	RMD		RMD	R[3]	
Testis	RM	RM	RMD	RMD	RMD	RMD	R	R
Epididymides	RM	RM	RMD	RMD	RMD	RMD	R	R
Prostate gland	RM	RM	RM	RMD		RMD	R	R
Seminal vesicle with coagulating gland				RMD		RMD	R	R
Mammary gland		R[1]M[1]		RMD[2]		RMD		
Ovary	R[1]M[1]	RM	RMD	RMD	RMD	RMD	R	R
Uterus with cervix	R[1]M[1]	RM	RMD	RMD	RMD	RMD	R	R
Vagina		RM		RM		RMD		R
Pituitary gland		R[1]M[1]	RM	RMD		RMD	R	
Adrenal gland	RM	RM	RMD	RMD	RMD	RMD	R	
Parathyroid gland	R[1]M[1]	R[1]M[1]	RMD	RMD	RMD	RMD		
Thyroid gland		RM		RMD		RMD	R	

[1] optional tissue [2] females only [3] only in pups

In addition to organ weight and microscopic examination, the 2-generation reproduction study provides information regarding the potential effect of a test compound on sperm production, quality, and motility; estrus cyclicity, time to mating and time to pregnancy; in utero development and effects on such parameters as anogenital distance; and sexual maturation of the offspring.

Specific information concerning (anti) androgenic / estrogenic potential and effects of the test item on sexual maturation is provided in assays conducted in immature male and female rats, using elements of the Hershberger and uterotrophic assays.

The standard repeat-dose studies available for isoflucypram thus provide sufficient data to allow determination of whether or not isoflucypram has effects on any endocrine modalities.

There were no effects in the rat, mouse, or dog 28-day or 90-day studies on either the weight (where measured) or the microscopic appearance of the pancreas, thymus, adrenal gland, testis, epididymides, prostate, seminal vesicles, ovaries, uterus, vagina, mammary gland, or pituitary gland.

In the dog one-year study, in males, there was a dose-related increase in the weight (both absolute and relative to brain weight) of the adrenal glands, a decrease in the absolute weight of the epididymides, and a decrease in the weight (both absolute and relative to brain weight) of the prostate. In females, there was a dose-related decrease in the weight (both absolute and relative to brain weight) of the thymus. However, these organ weight changes were not accompanied by any macroscopic or microscopic findings in either sex, and thus they are not indicative of any adverse effect.

In the rat 2-year study and mouse 18-month study, there was no effect on the weight of any of the organs listed in Table 2 (when organ weights were taken), nor was there any effect on either macroscopic or microscopic appearance.

In the rat 2-generation reproduction study, there was no effect in either the F0 or the F1 adults on weight or microscopic appearance of the adrenal glands, thymus, pituitary, ovaries, uterus, testes, seminal vesicles, or prostate, nor was there any effect in either generation on sperm parameters, estrus cyclicity, or any measure of either fertility or fecundity including time to mating and gestation length. In the F1 females, the age and body weight at vaginal opening was statistically significantly increased at the top dose only. There was however no alteration in these females in estrus cyclicity, time to mating, gestation length, or fertility or fecundity, and the number of ovarian follicles observed at sacrifice was not affected. The anogenital distance measured in F2 pups showed no change in either males or females.

In immature male rats, there were no effects on the weights of androgen-sensitive tissues after administration of isoflucypram alone or in combination with testosterone proprionate for 10 days. There was also no effect of administration of isoflucypram alone for 30 days on the timing of preputial separation. It can therefore be concluded that isoflucypram does not have any (anti-) androgenic effect in immature male rats.

In immature female rats, there was no effect on uterine weight in the uterotrophic assay. Furthermore, there was no effect on the timing of vaginal opening in immature female rats administered isoflucypram for 20 days beginning on postnatal day 19. In conclusion, isoflucypram does not have any (anti-) estrogenic or steroidogenic effects in immature female rats.

3.1.2. Evidence for adversity and general toxicity

There were no effects related to E, A, or S modalities in any of the repeat-dose studies; organ weight and macro- and microscopic appearance of these organs was unchanged, and there was no effect of treatment on function of any organ.

In the sexual matural study in immature male rats, body weight and / or body weight gain were statistically significantly reduced at the top dose tested (800 mg/kg bw/day), while decreased body weight and / or body weight gain was also observed at 400 mg/kg bw/day although it was not statistically significant at this dose level. There were occasional, inconsistent, statistically non-significant decreases in the weights of sex accessory tissues, however these were associated with the decreased body weight and thus are not indicative of an (anti-) androgenic effect.

In the sexual maturation study in immature female rats, the top dose tested (800 mg/kg bw/day) exceeded the Maximum Tolerated Dose, leading to mortalities, severe clinical signs, and decreased body weight and body weight gain. Due to these effects, uterine weight and vaginal opening were not examined in this group.

Table 3: Integrated lines of evidence regarding E, A, and S modalities for isoflucypram.

	Grouping	Line(s) of evidence	Species / cell line(s)	Expos., Weeks	Route of exposure	Effect dose Mg/kg bw/d or mg/L	Observed effects (positive and negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence	Modality
Integrated line of evidence for endocrine activity	EATS-mediated parameter	Sexual/reproductive parameters (EATS-mediated)	Rat	17-20 (2-gen)	Oral (dietary)	up to 109 (♂) / up to 112.5 (♀)	♀: ↑ age & BW at sexual maturation (VO)	Not supported by uterotrophic / vaginal opening assay	No estrogenic, androgenic or steroidogenic potential	E, A, S
			Rat	17-20 (2-gen)	Oral (dietary)	up to 41.6 (♂) / up to 44.5 (♀)	No effect	No indication of an endocrine effect		E, A, S
		Uterus (organ weight; gravid)	Rat	2	Oral (gavage)	625	No effect			E, A, S
			Rat	2	Oral (gavage)	125	No effect			E, A, S
			Rat	2	Oral (gavage)	25	No effect			E, A, S
			Rabbit	3	Oral (gavage)	500	No treatment-related effect			E, A, S
			Rabbit	3	Oral (gavage)	70 & 10	No effect			E, A, S
	In vivo mechanistic	Sex accessory tissues (organ weight)	Rat	30d	Oral (gavage)	800 & 400	↑ weight of ventral prostate & seminal vesicle (slight)	Considered not treatment-related as high variability	No androgenic potential	A
		Uterus (organ weight)	Rat	3d	Oral (gavage)	800 & 400	♀: no effect (only low magnitude changes, not SS, with high variability)		No estrogenic or androgenic potential	E, A
		Uterus (organ weight)	Rat	20d	Oral (gavage)	800 & 400	♀: no effect (only low magnitude changes, not SS, with high variability)		No estrogenic or androgenic potential	E, A

	Grouping	Line(s) of evidence	Species / cell line(s)	Expos., Weeks	Route of exposure	Effect dose Mg/kg bw/d or mg/L	Observed effects (positive and negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence	Modality
		VO	Rat	3d	Oral (gavage)	800 & 400	800 mg/kg/d: not interpretable (> MTD) 400 mg/kg/d: no effect	No effect observed	No estrogenic or androgenic potential	E, A
			Rat	20d	Oral (gavage)	800 & 400	800 mg/kg/d: not interpretable (> MTD) 400 mg/kg/d: no effect		No estrogenic or androgenic potential	E, A
		PPS	Rat	10d	Oral (gavage)	800	No effect		No estrogenic, androgenic or steroidogenic potential	E, A, S
			Rat	10d	Oral (gavage)	800 + TP & 400 + TP	No effect		No estrogenic, androgenic or steroidogenic potential	E, A, S
			Rat	30d	Oral (gavage)	800 & 400	No effect. Slight delay for 2/6 animals.	No difference for other animals: not treatment-related.	No estrogenic, androgenic or steroidogenic potential	E, A, S
		Sex accessory tissues (organ weight)	Rat	10d	Oral (gavage)	800	No effect	No effect observed	No effect on either preputial separation, weight of androgen-sensitive organs / sex accessory tissues, or timing of PS. No androgenic or an anti-androgenic activity.	E, A, S

	Grouping	Line(s) of evidence	Species / cell line(s)	Expos., Weeks	Route of exposure	Effect dose Mg/kg bw/d or mg/L	Observed effects (positive and negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence	Modality
			Rat	10d	Oral (gavage)	800 + TP & 400 + TP	No effect		No estrogenic, androgenic or steroidogenic potential	E, A, S
		Testes weight	Rat	30d	Oral (gavage)	800 & 400	No effect			E, A, S
Integrated line of evidence for adversity	Parameter sensitive to, but not diagnostic of, EATS	Abortions	Rabbit	3	Oral (gavage)	500	2 animals	Indicative of general toxicity	No indication of an endocrine effect	None
			Rabbit	3	Oral (gavage)	70 & 10	No effect	No effect		
		Adrenal gland (histology)	Rat	104	Oral (dietary)	18.6 (♂) / 46.6 (♀)	♂: focal medullary hyperplasia	Common finding in aging male Wistar rats, and although ↑ incidence, high p-values; therefore this finding was not considered treatment-related	No indication of an endocrine effect	
			Rat	104	Oral (dietary)	6.27 (♂) / 8.54 (♀)	No effect	No effect	No effect	
			Rat	104	Oral (dietary)	1.24 (♂) / 1.75 (♀)	No effect			
			Mouse	78	Oral (dietary)	147 (♂) / 190 (♀)	♂ & ♀: amyloid deposition (slight) at 18 months	Common finding in the aged mouse	Not an endocrine effect	
			Mouse	78	Oral (dietary)	29.0 (♂) / 38.1 (♀)	No effect	No effect	No effect	
			Mouse	78	Oral (dietary)	5.9 (♂) / 7.8 (♀)	No effect			
		Adrenal gland (organ weight)	Mouse	78	Oral (dietary)	147 (♂) / 190 (♀)	♂ & ♀: ↑ absolute and relative adrenal weights at 18 months	In absence of histopathological changes, considered incidental and not treatment-related	No indication of an endocrine effect	

	Grouping	Line(s) of evidence	Species / cell line(s)	Expos., Weeks	Route of exposure	Effect dose Mg/kg bw/d or mg/L	Observed effects (positive and negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence	Modality
			Mouse	78	Oral (dietary)	29.0 (♂) / 38.1 (♀)	No effect	No effect	No effect	
		Cesarean sections	Rat	2	Oral (gavage)	625	No effect			
			Rat	2	Oral (gavage)	125	Incomplete ossification (not SS)	Delayed ossification, commonly seen spontaneous variations. Considered to represent only a slight and transient delay in fetal development with no adverse long-term consequences.	No indication of an endocrine effect	
			Rat	2	Oral (gavage)	25	No effect	No effect	No effect	
			Rabbit	3	Oral (gavage)	500	No treatment-related effect			
			Rabbit	3	Oral (gavage)	70 & 10	No effect			
		Cholesterol level	Rat	4	Oral (dietary)	241 (♂) / 285 (♀)	♂: ↑ total cholesterol ♀: no effect	Related to general mode of action	Indicative of a CAR / PXR, not an endocrine, mode of action	
			Dog	4	Oral (dietary)	76.9 (♂) / 90.2 (♀)	↓ cholesterol			
			Rat	13	Oral (dietary)	63.5 (♂) / 80.9 (♀)	♀: ↑ total cholesterol			

	Grouping	Line(s) of evidence	Species / cell line(s)	Expos., Weeks	Route of exposure	Effect dose Mg/kg bw/d or mg/L	Observed effects (positive and negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence	Modality
		Fetal malformations / variations	Rat	2	Oral (gavage)	625	No treatment-related malformations ↑ distended bladders and renal pelvis dilatation Delayed ossifications, suggestive of a moderate delay in the fetal development No effects on genital abnormalities	No indication of an endocrine effect	No indication of an endocrine effect	
			Rat	2	Oral (gavage)	125	No effect	No effect	no endocrine effect	
			Rat	2	Oral (gavage)	25	No effect			
			Rabbit	3	Oral (gavage)	500	No treatment-related effect			
			Rabbit	3	Oral (gavage)	70 & 10	No effect			
		Sexual/reproductive parameters	Rat	17-20 (2-gen)	Oral (dietary)	up to 109 (♂) / up to 112.5 (♀)	F1: Slight shift in gestation length	Not consistent across generations	No indication of an endocrine effect	
			Rat	17-20 (2-gen)	Oral (dietary)	up to 41.6 (♂) / up to 44.5 (♀)	No effect	No effect	No endocrine effect	
Evidence of general toxicity	Kidney	Kidney (histology)	Rat	4	Oral (dietary)	241 (♂) / 285 (♀)	♂: tubular hyaline droplets + increased incidence of bilateral basophilic tubules	Hyaline droplet nephropathy is a recognized lesion of the male rat kidney. Not relevant to humans.	No endocrine effect	

	Grouping	Line(s) of evidence	Species / cell line(s)	Expos., Weeks	Route of exposure	Effect dose Mg/kg bw/d or mg/L	Observed effects (positive and negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence	Modality
			Rat	4	Oral (dietary)	83.3 (♂) / 86.5 (♀)	♂: tubular hyaline droplets + increased incidence of bilateral basophilic tubules			
			Rat	4	Oral (dietary)	22.8 (♂) / 25.6 (♀)	♂: tubular hyaline droplets (3/5 animals). Not associated with basophilic tubules so considered not adverse			
			Rat	13	Oral (dietary)	63.5 (♂) / 80.9 (♀)	♂: hyaline droplets in proximal tubules and bilateral basophilic tubules			
			Rat	13	Oral (dietary)	18.4 (♂) / 21.9 (♀)	♂: hyaline droplets in proximal tubules (within HCD)			
			Mouse	79	Oral (dietary)	148 (♂) / 190 (♀)	♂: hyaline casts, tubule dilation in the medulla, and focal tubule basophilia at 18 months ♀: amyloid deposition at 18 months	Evidence of a potential renal effect following lifetime administration at high doses		
			Mouse	79	Oral (dietary)	29.0 (♂) / 38.1 (♀)	No effect			
			Mouse	79	Oral (dietary)	5.9 (♂) / 7.8 (♀)	No effect			

	Grouping	Line(s) of evidence	Species / cell line(s)	Expos., Weeks	Route of exposure	Effect dose Mg/kg bw/d or mg/L	Observed effects (positive and negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence	Modality
		Kidney (weight)	Mouse	78	Oral (dietary)	147 (♂) / 190 (♀)	♂:↑ absolute and relative kidney weights at 18 months			
			Mouse	78	Oral (dietary)	29.0 (♂) / 38.1 (♀)	No effect			
			Mouse	78	Oral (dietary)	5.9 (♂) / 7.8 (♀)	No effect			

Table 4: Integrated lines of evidence regarding the T modality for isoflucypram.

	Grouping	Line(s) of evidence	Species / cell line(s)	Expos., Weeks	Route of exposure	Effect dose Mg/kg bw/d or mg/L	Observed effects (positive and negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence	Modality
Integrated line of evidence for endocrine activity	In vivo mechanistic	Liver (organ weight associated with thyroid endpoint)	Rat	4	Oral (dietary)	241 (♂) / 285 (♀)	♂ & ♀: ↑ absolute and relative liver weights	Increased liver weight indicative of a proliferative effect of treatment on the liver, leading to a potential secondary effect on the thyrid	These findings clearly demonstrate that the liver is the primary target organ of isoflucypram, that the compound acts on the liver through induction of hepatic Phase I and Phase II enzymes and hepatocellular porliferation, and that in the rat but not the mouse or dog, this is accompanied by an increase in thyroid follicular cell hypertrophy and thyroid weight, by a slight decrease in the concentrations	T
			Rat	4	Oral (dietary)	83.3 (♂) / 86.5 (♀)	♂ & ♀: ↑ absolute and relative liver weights			
			Rat	13	Oral (dietary)	63.5 (♂) / 80.9 (♀)	♂: ↑ relative liver weights ♀: ↑ absolute and relative liver weights			
			Rat	104	Oral (dietary)	18.6 (♂) / 46.6 (♀)	♀: ↑ absolute and relative liver weights at 12 months, ↑ relative liver weights at 24 months			
			Rat	104	Oral (dietary)	6.27 (♂) / 8.54 (♀)	No effect			
			Rat	104	Oral (dietary)	1.24 (♂) / 1.75 (♀)	No effect			
	EATS-mediated parameter	Thyroid (histology)	Rat	4	Oral (dietary)	241 (♂) / 285 (♀)	♀ & ♂: Follicular cell hypertrophy (minimal to slight)	Indicates that the thyroid effect observed after isoflucypram administration is specific to the rat, as no effects were		
			Mouse	4	Oral (dietary)	330 (♂) / 374 (♀)	No effect			
			Dog	4	Oral (dietary)	76.9 (♂) / 90.2 (♀)	No effect			

	Grouping	Line(s) of evidence	Species / cell line(s)	Expos., Weeks	Route of exposure	Effect dose Mg/kg bw/d or mg/L	Observed effects (positive and negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence	Modality
			Rat	13	Oral (dietary)	63.5 (♂) / 80.9 (♀)	♀ & ♂: follicular cell hypertrophy (minimal) and of colloid alteration	observed in either mouse or dog. These degenerative, non-neoplastic findings can be associated with the slight and prolonged thyroid gland stimulation and therefore were not considered to be adverse. Commonly seen in aging Wistar rats + not associated with any signs of hyperparathyroidism or hypercalcemia and therefore was not considered not treatment-related Commonly seen in aging Wistar rats + not associated with any signs of hyperparathyroidism or hypercalcemia and therefore was not	of T3 and / or T4, and by an increase in TSH concentration and / or transcription of TSHβ in the pituitary. These findings taken together clearly show that isoflucypram acts on the rat thyroid via a rat-specific, liver-mediated CAR/PXR mode of action and is not a primary endocrine disruptor.	
			Mouse	13	Oral (dietary)	168 (♂) / 207 (♀)	No effect			
			Mouse	13	Oral (dietary)	51.0 (♂) / 59.8 (♀)	No effect			
			Mouse	13	Oral (dietary)	17.0 (♂) / 19.5 (♀)	No effect			
			Dog	13	Oral (dietary)	50.4 (♂) / 54.0 (♀)	No effect			
			Dog	13	Oral (dietary)	15.9 (♂) / 16.2 (♀)	No effect			
			Dog	13	Oral (dietary)	5.5 (♂) / 5.5 (♀)	No effect			
			Dog	52	Oral (dietary)	60.2 (♂) / 49.8 (♀)	No effect			
			Dog	52	Oral (dietary)	18.8 (♂) / 17.6 (♀)	No effect			
			Dog	52	Oral (dietary)	4.2 (♂) / 4.2 (♀)	No effect			
			Rat	104	Oral (dietary)	18.6 (♂) / 46.6 (♀)	♂ & ♀: colloid alteration (SS in ♀ only) ♂: diffuse pigmentation of the follicular cells			
			Rat	104	Oral (dietary)	18.6 (♂) / 46.6 (♀)	♂: diffuse C-cell hyperplasia			

	Grouping	Line(s) of evidence	Species / cell line(s)	Expos., Weeks	Route of exposure	Effect dose Mg/kg bw/d or mg/L	Observed effects (positive and negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence	Modality
			Rat	104	Oral (dietary)	6.27 (♂) / 8.54 (♀)	♂: diffuse C-cell hyperplasia	considered not treatment-related		
			Rat	104	Oral (dietary)	1.24 (♂) / 1.75 (♀)	No effect			
			Rat	104	Oral (dietary)	1.24 (♂) / 1.75 (♀)	No effect			
			Mouse	78	Oral (dietary)	147 (♂) / 190 (♀)	♂ & ♀: amyloid deposition (slight) at 18 months			
			Mouse	78	Oral (dietary)	29.0 (♂) / 38.1 (♀)	No effect			
			Mouse	78	Oral (dietary)	5.9 (♂) / 7.8 (♀)	No effect			
			Rat	2	Oral (gavage)	625	Thyroid follicular hypertrophy (1 animal)			
			Rat	2	Oral (gavage)	125	No effect			
			Rat	2	Oral (gavage)	25	No effect			
			Rat	1	Oral (dietary)	67	♀: no microscopic findings but ↑ follicular cell proliferation (reversible)			
			Rat	1	Oral (dietary)	36	No effect			
			Rat	1	Oral (dietary)	12	No effect			

	Grouping	Line(s) of evidence	Species / cell line(s)	Expos., Weeks	Route of exposure	Effect dose Mg/kg bw/d or mg/L	Observed effects (positive and negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence	Modality
			Rat	1	Oral (dietary)	6 & 2.4	No effect			
			Mouse	1	Oral (dietary)	224, 94.1 & 43.9	No effect			
			Mouse	1	Oral (dietary)	18.6 & 8.87	No effect			
			Rat	4	Oral (dietary)	69	♀: Follicular cell hypertrophy: diffuse (minimal) Associated with ↑ cell proliferation.			
			Rat	4	Oral (dietary)	37, 12, 6 & 2.4	No microscopic findings Associated with ↑ cell proliferation @ 37 mg/kg/d only.			
		Thyroid (organ weight)	Rat	4	Oral (dietary)	241 (♂) / 285 (♀)	♂ & ♀: ↑ absolute and relative thyroid weights			
			Mouse	4	Oral (dietary)	330 (♂) / 374 (♀)	No effect			
			Dog	4	Oral (dietary)	76.9 (♂) / 90.2 (♀)	No effect			
			Rat	13	Oral (dietary)	63.5 (♂) / 80.9 (♀)	♂: ↑ relative thyroid weights			
			Rat	13	Oral (dietary)	18.4 (♂) / 21.9 (♀)	No effect			
			Rat	13	Oral (dietary)	6.34 (♂) / 7.92 (♀)	No effect			

	Grouping	Line(s) of evidence	Species / cell line(s)	Expos., Weeks	Route of exposure	Effect dose Mg/kg bw/d or mg/L	Observed effects (positive and negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence	Modality
			Mouse	13	Oral (dietary)	168 (♂) / 207 (♀)	No effect			
			Mouse	13	Oral (dietary)	51.0 (♂) / 59.8 (♀)	No effect			
			Mouse	13	Oral (dietary)	17.0 (♂) / 19.5 (♀)	No effect			
			Dog	13	Oral (dietary)	50.4 (♂) / 54.0 (♀)	No effect			
			Dog	13	Oral (dietary)	15.9 (♂) / 16.2 (♀)	No effect			
			Dog	13	Oral (dietary)	5.5 (♂) / 5.5 (♀)	No effect			
			Dog	52	Oral (dietary)	60.2 (♂) / 49.8 (♀)	No effect			
			Dog	52	Oral (dietary)	18.8 (♂) / 17.6 (♀)	No effect			
			Dog	52	Oral (dietary)	4.2 (♂) / 4.2 (♀)	No effect			
			Rat	104	Oral (dietary)	18.6 (♂) / 46.6 (♀)	No effect			
			Rat	104	Oral (dietary)	6.27 (♂) / 8.54 (♀)	No effect			
			Rat	104	Oral (dietary)	1.24 (♂) / 1.75 (♀)	No effect			
			Mouse	78	Oral (dietary)	147 (♂) / 190 (♀)	No effect			
			Mouse	78	Oral (dietary)	29.0 (♂) / 38.1 (♀)	No effect			

	Grouping	Line(s) of evidence	Species / cell line(s)	Expos., Weeks	Route of exposure	Effect dose Mg/kg bw/d or mg/L	Observed effects (positive and negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence	Modality
			Mouse	78	Oral (dietary)	5.9 (♂) / 7.8 (♀)	No effect			
			Rat	17-20 (2-gen)	Oral (dietary)	up to 109 (♂) / up to 112.5 (♀)	F0 ♂: ↑ thyroid weight			
			Rat	17-20 (2-gen)	Oral (dietary)	up to 41.6 (♂) / up to 44.5 (♀)	No effect			
			Rat	2	Oral (gavage)	625	No effect			
			Rat	2	Oral (gavage)	125	No effect			
			Rat	2	Oral (gavage)	25	No effect			
			Rat	1	Oral (dietary)	67	No effect			
			Rat	1	Oral (dietary)	36	No effect			
			Rat	1	Oral (dietary)	12	No effect			
			Rat	1	Oral (dietary)	6 & 2.4	No effect			
			Mouse	1	Oral (dietary)	224, 94.1 & 43.9	No effect			
			Mouse	1	Oral (dietary)	18.6 & 8.87	No effect			
			Rat	4	Oral (dietary)	69	No effect			
			Rat	4	Oral (dietary)	37, 12, 6 & 2.4	No effect			

	Grouping	Line(s) of evidence	Species / cell line(s)	Expos., Weeks	Route of exposure	Effect dose Mg/kg bw/d or mg/L	Observed effects (positive and negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence	Modality
	In vivo mechanistic Liver	Hepatic enzyme induction	Rat	1	Oral (dietary)	67	↑ activity of T4-UDPGT (reversible)	Indicative of the CAR/PXR mode of action in the rodent		
			Rat	1	Oral (dietary)	36	↑ activity of T4-UDPGT (not SS)			
			Rat	4	Oral (dietary)	84.8 & 26.4	♀: ↑ T4-UDPGT, dose-related			
			Rat	4	Oral (dietary)	69	♀: ↑ T4-UDPGT			
			Rat	4	Oral (dietary)	37, 12, 6 & 2.4	♀: ↑ T4-UDPGT			
		Thyroid hormone concentration	Rat	1	Oral (dietary)	67	♀: ↑ TSH (slight, not SS). ↑ Tsh β gene in pituitary (slight, not SS)	Indicative of an effect on thyroid hormone concentration only at doses where hepatic Phase II enzyme induction was also observed		
			Rat	1	Oral (dietary)	36	♀: ↑ T3 (slight, SS, no clear dose-effect relationship). ↑ Tsh β in pituitary (slight, not SS)			
			Rat	1	Oral (dietary)	12	♀: ↑ T3 (slight, SS, no clear dose-effect relationship)			
			Rat	1	Oral (dietary)	6 & 2.4	No effect			

	Grouping	Line(s) of evidence	Species / cell line(s)	Expos., Weeks	Route of exposure	Effect dose Mg/kg bw/d or mg/L	Observed effects (positive and negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence	Modality
			Rat	4	Oral (dietary)	84.8 & 26.4	♀: no effect on T3 & T4 ↑ TSH ↑ Tshβ gene in pituitary			
			Rat	4	Oral (dietary)	69	♀: no effect on T3 & T4 ↑ TSH (dose-related)			
			Rat	4	Oral (dietary)	37, 12, 6 & 2.4	♀: no effect on T3 & T4 ↑ TSH (dose-related, not SS)			
		Hepatic enzyme induction	Rat	4	Oral (dietary)	241 (♂) / 285 (♀)	♂: ↑ BROD, PROD, bilirubin- and 4-nitrophenol-UDPGT. Slight ↑ P450 and EROD. ♀: ↑ BROD, bilirubin- and 4-nitrophenol-UDPGT. Slight ↑ PROD.	Indicative of the CAR/PXR mode of action in the rodent		
			Rat	4	Oral (dietary)	241 (♂) / 285 (♀)	♂ & ♀: ↑ T4-UDPGT.			

	Grouping	Line(s) of evidence	Species / cell line(s)	Expos., Weeks	Route of exposure	Effect dose Mg/kg bw/d or mg/L	Observed effects (positive and negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence	Modality
			Rat	4	Oral (dietary)	83.3 (♂) / 86.5 (♀)	♂: ↑ BROD, bilirubin- and 4-nitrophenol-UDPGT. Slight ↑ P450, EROD, and PROD. ♀: ↑ BROD, bilirubin- and 4-nitrophenol-UDPGT. Slight ↑ PROD.			
			Rat	4	Oral (dietary)	83.3 (♂) / 86.5 (♀)	♂ & ♀: ↑ T4-UDPGT.			
			Rat	4	Oral (dietary)	22.8 (♂) / 25.6 (♀)	♂: no induction. ♀: Slight ↑ bilirubin-UDPGT (low magnitude, considered not adverse)			
			Rat	1	Oral (dietary)	67	♀: ↑ activities of bilirubin-UDPGT, 4-nitrophenol UDPGT, and P450 (reversible) ↓ expression of cyp4a1 gene ↑ expression of Cyp2b1 gene (but no ↑ of BROD activ), Cyp3a23, and Ugt1a1, Ugt1a6, Ugt2b1 and Gadd45b genes (reversible)			

	Grouping	Line(s) of evidence	Species / cell line(s)	Expos., Weeks	Route of exposure	Effect dose Mg/kg bw/d or mg/L	Observed effects (positive and negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence	Modality
			Rat	1	Oral (dietary)	36	♀: ↑ activities of bilirubin-UDPGT (reversible) ↑ expression of Cyp2b1 gene (but no ↑ of BROD activ), Cyp3a23, and Ugt1a1, Ugt1a6, Ugt2b1 and Gadd45b genes			
			Rat	1	Oral (dietary)	12	♀: ↑ expression of Cyp2b1 gene (but no ↑ of BROD activ), Ugt1a1, Ugt2b1 and Gadd45b genes			
			Rat	1	Oral (dietary)	12	No effect			
			Rat	1	Oral (dietary)	6 & 2.4	♀: ↑ expression of Cyp2b1 gene (but no ↑ of BROD activ) and Ugt2b1 gene, @ 6 mg/kg/d. No effect @ 2.4 mg/kg/d.			
			Rat	1	Oral (dietary)	6 & 2.4	No effect			

	Grouping	Line(s) of evidence	Species / cell line(s)	Expos., Weeks	Route of exposure	Effect dose Mg/kg bw/d or mg/L	Observed effects (positive and negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence	Modality
			Mouse	1	Oral (dietary)	224, 94.1 & 43.9	♀: ↑ activities of bilirubin-UDPGT, BQ (except @ 43.9 mg/kg/d), PROD and P450 (except @ 43.9 mg/kg/d) (reversible). ↑ expression of Cyp1a2 (except @ 43.9 mg/kg/d), Cyp2b10, Cyp3a11, Ugt2b5 and Ugt1a1 (except @ 43.9 mg/kg/d) genes, and at 231 mg/kg/d only, Sult1d1, and Gadd45a genes (reversible)			
			Mouse	1	Oral (dietary)	18.6 & 8.87	♀: ↑ activity of PROD, ↑ bilirubin-UDPGT (only @18.6 mg/kg/d). ↑ expression of Cyp2b10 & Cyp3a11 genes, and Ugt2b5 gene (only @ 18.6 mg/kg/d).			

	Grouping	Line(s) of evidence	Species / cell line(s)	Expos., Weeks	Route of exposure	Effect dose Mg/kg bw/d or mg/L	Observed effects (positive and negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence	Modality
			Rat	4	Oral (dietary)	84.8 & 26.4	♀: ↑ BROD and bilirubin-UDPGT, dose-related. Dose-related ↑ in transcription of a number of Phase I and Phase II genes (Cyp1a1, Cyp2b1, Cyp3a3, Por, Ugt1a6, Ugt2b1, Sult2a2, Ephx1, Gstm4). ↓ Cyp4a1			
			Rat	4	Oral (dietary)	69	♀: ↑ BROD, 4-nitrophenol-UDPGT and bilirubin-UDPGT Dose-related ↑ in transcription of a number of Phase I and Phase II genes (Cyp1a1, Cyp2b1, Cyp3a23, Ugt1a1, Ugt2b1, and Gadd45b). ↓ Cyp4a1			

	Grouping	Line(s) of evidence	Species / cell line(s)	Expos., Weeks	Route of exposure	Effect dose Mg/kg bw/d or mg/L	Observed effects (positive and negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence	Modality
			Rat	4	Oral (dietary)	37, 12, 6 & 2.4	♀: @ 37 mg/kg/d only: ↑ BROD, 4-nitrophenol-UDPGT and bilirubin-UDPGT Dose-related ↑ in transcription of a number of Phase I and Phase II genes (Cyp1a1, Cyp2b1, Cyp3a23, Ugt1a1, Ugt2b1, and Gadd45b), except @ 2.4 mg/kg/d			
Evidence of toxicological mode of action		Liver (histology)	Rat	4	Oral (dietary)	241 (♂) / 285 (♀)	♀ & ♂: Panlobular hypertrophy and hepatocellular periportal microvacuolations	Indicates that liver is the primary target organ in all species tested		
			Rat	4	Oral (dietary)	83.3 (♂) / 86.5 (♀)	Panlobular hypertrophy (1 ♀ animal)			
			Mouse	4	Oral (dietary)	330 (♂) / 374 (♀)	Centrilobular hepatocellular hypertrophy, hepatocellular necrotic foci, and focal hepatocellular single cell necrosis (♀ & ♂)			

	Grouping	Line(s) of evidence	Species / cell line(s)	Expos., Weeks	Route of exposure	Effect dose Mg/kg bw/d or mg/L	Observed effects (positive and negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence	Modality
			Mouse	4	Oral (dietary)	133 (♂) / 149 (♀)	Hepatocellular necrotic foci (slight, 1 ♂), and focal hepatocellular single cell necrosis (minimal, 2/5 ♂)			
			Mouse	4	Oral (dietary)	32 (♂) / 41 (♀)	No effect			
			Dog	4	Oral (dietary)	76.9 (♂) / 90.2 (♀)	♂ & ♀: centrilobular to panlobular hepatocellular hypertrophy, ↓ hepatocellular glycogen accumulation ♀: brown pigmen accumulation in Kupffer cells			
			Dog	4	Oral (dietary)	37.7 (♂) / 36.5 (♀)	No effect			
			Dog	4	Oral (dietary)	12.7 (♂) / 11.3 (♀)	No effect			
			Rat	13	Oral (dietary)	63.5 (♂) / 80.9 (♀)	♀: periportal to panlobular hepatocellular hypertrophy (minimal to slight)			

	Grouping	Line(s) of evidence	Species / cell line(s)	Expos., Weeks	Route of exposure	Effect dose Mg/kg bw/d or mg/L	Observed effects (positive and negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence	Modality
			Mouse	13	Oral (dietary)	168 (♂) / 207 (♀)	♀ & ♂: ↑ centrilobular hepatocellular vacuolation + ↓ diffuse hepatocellular vacuolation = loss of vacuolation in the periportal area of the hepatic lobule.			
			Mouse	13	Oral (dietary)	51.0 (♂) / 59.8 (♀)	♀: ↑ centrilobular hepatocellular vacuolation + ↓ diffuse hepatocellular vacuolation			
			Mouse	13	Oral (dietary)	17.0 (♂) / 19.5 (♀)	No effect			
			Dog	13	Oral (dietary)	50.4 (♂) / 54.0 (♀)	♂: centrilobular hepatocellular hypertrophy + cytoplasmic changes (eosinophilic intracytoplasmic vacuoles)			
			Dog	13	Oral (dietary)	15.9 (♂) / 16.2 (♀)	No effect			
			Dog	13	Oral (dietary)	5.5 (♂) / 5.5 (♀)	No effect			

	Grouping	Line(s) of evidence	Species / cell line(s)	Expos., Weeks	Route of exposure	Effect dose Mg/kg bw/d or mg/L	Observed effects (positive and negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence	Modality
			Dog	52	Oral (dietary)	60.2 (♂) / 49.8 (♀)	♂ & ♀: centrilobular hepatocellular hypertrpohy (minimal to moderate) + Kupffer cell pigmentation ♀: single cell necrosis, and cytoplasmic hepatocellular changes (eosinophilic intracytoplasmic inclusions)			
			Dog	52	Oral (dietary)	18.8 (♂) / 17.6 (♀)	♂ & ♀: centrilobular hepatocellular hypertrpohy (minimal)			
			Dog	52	Oral (dietary)	4.2 (♂) / 4.2 (♀)	No effect			
			Rat	104	Oral (dietary)	18.6 (♂) / 46.6 (♀)	No effect			
			Rat	104	Oral (dietary)	6.27 (♂) / 8.54 (♀)	No effect			
			Rat	104	Oral (dietary)	1.24 (♂) / 1.75 (♀)	No effect			

	Grouping	Line(s) of evidence	Species / cell line(s)	Expos., Weeks	Route of exposure	Effect dose Mg/kg bw/d or mg/L	Observed effects (positive and negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence	Modality
			Mouse	78	Oral (dietary)	147 (♂) / 190 (♀)	♂: multinucleated hepatocytes at 12 & 18 months, single cell necrosis at 18 months ♀: bile duct hyperplasia at 12 and 18 months, hepatocellular necrotic foci at 18 months, and ↓ diffuse hepatocellular vacuolation at 18 months ♂ & ♀: amyloid deposition (slight) at 18 months			
			Mouse	78	Oral (dietary)	29.0 (♂) / 38.1 (♀)	No effect			
			Mouse	78	Oral (dietary)	5.9 (♂) / 7.8 (♀)	No effect			
			Rat	2	Oral (gavage)	625	♀: hepatocellular centrilobular hypertrophy (minimal to slight) follicular cell hypertrophy (minimal, 1 animal)			
			Rat	2	Oral (gavage)	125	No effect			

	Grouping	Line(s) of evidence	Species / cell line(s)	Expos., Weeks	Route of exposure	Effect dose Mg/kg bw/d or mg/L	Observed effects (positive and negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence	Modality
			Rat	2	Oral (gavage)	25	No effect			
			Rat	1	Oral (dietary)	67	♀: No microscopic findings but ↑ cell proliferation (periportal) (reversible)			
			Rat	1	Oral (dietary)	36	No effect			
			Rat	1	Oral (dietary)	12	No effect			
			Rat	1	Oral (dietary)	6 & 2.4	No effect			
			Mouse	1	Oral (dietary)	224, 94.1 & 43.9	♀: no microscopic findings but ↑ cell proliferation (unclear, not dose-related, not SS)			
			Mouse	1	Oral (dietary)	18.6 & 8.87	No effect			
			Rat	4	Oral (dietary)	69	♀: Hepatocellular hypertrophy, centrilobular to panlobular, diffuse (minimal) No cell proliferation			
			Rat	4	Oral (dietary)	37, 12, 6 & 2.4	No microscopic findings No cell proliferation			

	Grouping	Line(s) of evidence	Species / cell line(s)	Expos., Weeks	Route of exposure	Effect dose Mg/kg bw/d or mg/L	Observed effects (positive and negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence	Modality
		Liver (organ weight)	Mouse	4	Oral (dietary)	330 (♂) / 374 (♀)	♂ & ♀: ↑ absolute and relative liver weights			
			Mouse	4	Oral (dietary)	133 (♂) / 149 (♀)	♀: ↑ absolute and relative liver weights			
			Mouse	4	Oral (dietary)	32 (♂) / 41 (♀)	No effect			
			Dog	4	Oral (dietary)	76.9 (♂) / 90.2 (♀)	↑ absolute and relative liver weights			
			Dog	4	Oral (dietary)	37.7 (♂) / 36.5 (♀)	No effect			
			Dog	4	Oral (dietary)	12.7 (♂) / 11.3 (♀)	No effect			
			Mouse	13	Oral (dietary)	168 (♂) / 207 (♀)	♀ & ♂: ↑ absolute and relative liver weights			
			Mouse	13	Oral (dietary)	51.0 (♂) / 59.8 (♀)	♂: ↑ absolute and relative liver weights			
			Mouse	13	Oral (dietary)	17.0 (♂) / 19.5 (♀)	No effect			
			Dog	13	Oral (dietary)	50.4 (♂) / 54.0 (♀)	♂: ↑ absolute and relative liver weights			
			Dog	13	Oral (dietary)	15.9 (♂) / 16.2 (♀)	No effect			
			Dog	13	Oral (dietary)	5.5 (♂) / 5.5 (♀)	No effect			

	Grouping	Line(s) of evidence	Species / cell line(s)	Expos., Weeks	Route of exposure	Effect dose Mg/kg bw/d or mg/L	Observed effects (positive and negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence	Modality
			Dog	52	Oral (dietary)	60.2 (♂) / 49.8 (♀)	♂: ↑ absolute and relative liver weights			
			Dog	52	Oral (dietary)	18.8 (♂) / 17.6 (♀)	No effect			
			Dog	52	Oral (dietary)	4.2 (♂) / 4.2 (♀)	No effect			
			Mouse	78	Oral (dietary)	147 (♂) / 190 (♀)	♂ & ♀: ↑ absolute and relative liver weights at 12 months, ♂:↑ absolute and relative liver weights at 18 months			
			Mouse	78	Oral (dietary)	29.0 (♂) / 38.1 (♀)	No effect			
			Mouse	78	Oral (dietary)	5.9 (♂) / 7.8 (♀)	No effect			
			Rat	17-20 (2-gen)	Oral (dietary)	up to 109 (♂) / up to 112.5 (♀)	F0, F1 & F2 ♂ &/or ♀: ↑ liver weight			
			Rat	17-20 (2-gen)	Oral (dietary)	up to 41.6 (♂) / up to 44.5 (♀)	F1 & F2 ♀: ↑ liver weight			
			Rat	2	Oral (gavage)	625	♀: ↑ liver weight			
			Rat	2	Oral (gavage)	125	No effect			
			Rat	2	Oral (gavage)	25	No effect			

	Grouping	Line(s) of evidence	Species / cell line(s)	Expos., Weeks	Route of exposure	Effect dose Mg/kg bw/d or mg/L	Observed effects (positive and negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence	Modality
			Rabbit	3	Oral (gavage)	500	♀: ↑ liver weight			
			Rabbit	3	Oral (gavage)	70 & 10	No effect			
			Rat	1	Oral (dietary)	67	♀: ↑ relative liver weight			
			Rat	1	Oral (dietary)	36	No effect			
			Rat	1	Oral (dietary)	12	No effect			
			Rat	1	Oral (dietary)	6 & 2.4	No effect			
			Mouse	1	Oral (dietary)	224, 94.1 & 43.9	♀: ↑ absolute & relative liver weight			
			Mouse	1	Oral (dietary)	18.6 & 8.87	No effect			
			Rat	4	Oral (dietary)	84.8 & 26.4	♀: ↑ absolute & relative liver wt			
			Rat	4	Oral (dietary)	69	♀: ↑ absolute & relative liver wt			
			Rat	4	Oral (dietary)	37, 12, 6 & 2.4	♀: ↑ relative liver wt @ 37 mg/kg/d			
			Rat	10d	Oral (gavage)	800	♂: ↑ absolute & relative liver wt			
			Rat	10d	Oral (gavage)	800 + TP & 400 + TP	♂: ↑ absolute & relative liver wt			

	Grouping	Line(s) of evidence	Species / cell line(s)	Expos., Weeks	Route of exposure	Effect dose Mg/kg bw/d or mg/L	Observed effects (positive and negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence	Modality
			Rat	30d	Oral (gavage)	800 & 400	♂: ↑ absolute & relative liver wt (not SS)			
			Rat	20d	Oral (gavage)	800 & 400	♀: ↑ absolute & relative liver wt			
		Clinical chemistry	Rat	4	Oral (dietary)	241 (♂) / 285 (♀)	♂: ↑ total protein, albumin, ↓ total bilirubin ♀: ↓ total bilirubin	↓ total bilirubin considered not to be adverse effect of the test item as it does not represent any functional impairment in the animal, however it is indicative of the mode of action of the compound in the liver		
			Rat	4	Oral (dietary)	83.3 (♂) / 86.5 (♀)	♂ & ♀: ↓ total bilirubin			
			Rat	4	Oral (dietary)	22.8 (♂) / 25.6 (♀)	♂ & ♀: ↓ total bilirubin			
			Mouse	4	Oral (dietary)	330 (♂) / 374 (♀)	♂ & ♀: ↑ ASAT, ALAT, AP ♀: ↓ total bilirubin			
			Mouse	4	Oral (dietary)	133 (♂) / 149 (♀)	♂ & ♀: ↑ ASAT ♀: ↓ total bilirubin			
			Mouse	4	Oral (dietary)	32 (♂) / 41 (♀)	♀: ↓ total bilirubin			
			Dog	4	Oral (dietary)	76.9 (♂) / 90.2 (♀)	↑ AP			
			Dog	4	Oral (dietary)	37.7 (♂) / 36.5 (♀)	♀: ↑ AP. Considered not adverse as not associated w microscopic changes			
			Dog	4	Oral (dietary)	12.7 (♂) / 11.3 (♀)	No effect			

	Grouping	Line(s) of evidence	Species / cell line(s)	Expos., Weeks	Route of exposure	Effect dose Mg/kg bw/d or mg/L	Observed effects (positive and negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence	Modality
			Rat	13	Oral (dietary)	63.5 (♂) / 80.9 (♀)	♀ & ♂: ↓ total bilirubin ♂: cellular casts in the urine (associated w micro findings in kidney)			
			Rat	13	Oral (dietary)	18.4 (♂) / 21.9 (♀)	♀: ↓ total bilirubin ♂ (1 animal): cellular casts in the urine (within HCD)			
			Rat	13	Oral (dietary)	6.34 (♂) / 7.92 (♀)	♀: ↓ total bilirubin			
			Mouse	13	Oral (dietary)	168 (♂) / 207 (♀)	♀ & ♂: ↓ total bilirubin			
			Mouse	13	Oral (dietary)	51.0 (♂) / 59.8 (♀)	♀ & ♂: ↓ total bilirubin			
			Mouse	13	Oral (dietary)	51.0 (♂) / 59.8 (♀)	♀ & ♂: ↓ total bilirubin (tendency)			
			Dog	13	Oral (dietary)	50.4 (♂) / 54.0 (♀)	♀ & ♂: ↑ AP, ↓ total bilirubin			
			Dog	13	Oral (dietary)	15.9 (♂) / 16.2 (♀)	♀ & ♂: ↑ AP, ♂: ↓ total bilirubin			
			Dog	13	Oral (dietary)	5.5 (♂) / 5.5 (♀)	♀ & ♂: ↑ AP, ♂: ↓ total bilirubin			
			Dog	52	Oral (dietary)	60.2 (♂) / 49.8 (♀)	♀ & ♂: ↑ AP			
			Dog	52	Oral (dietary)	18.8 (♂) / 17.6 (♀)	♀ & ♂: ↑ AP			

	Grouping	Line(s) of evidence	Species / cell line(s)	Expos., Weeks	Route of exposure	Effect dose Mg/kg bw/d or mg/L	Observed effects (positive and negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence	Modality
			Dog	52	Oral (dietary)	4.2 (♂) / 4.2 (♀)	No effect			
			Rat	104	Oral (dietary)	18.6 (♂) / 46.6 (♀)	♀: ↓ total bilirubin			
			Rat	104	Oral (dietary)	6.27 (♂) / 8.54 (♀)	No effect			
			Rat	104	Oral (dietary)	1.24 (♂) / 1.75 (♀)	No effect			
			Rat	17-20 (2-gen)	Oral (dietary)	up to 109 (♂) / up to 112.5 (♀)	No effect			
			Rat	17-20 (2-gen)	Oral (dietary)	up to 41.6 (♂) / up to 44.5 (♀)	No effect			
			Rat	2	Oral (gavage)	625	♀: ↓ total bilirubin, ↓ AP			
			Rat	2	Oral (gavage)	125	♀: ↓ total bilirubin			
			Rat	2	Oral (gavage)	25	♀: ↓ total bilirubin			
			Rat	4	Oral (dietary)	69	♀: ↓ bilirubin (dose-related)			
			Rat	4	Oral (dietary)	37, 12, 6 & 2.4	♀: ↓ bilirubin (dose-related, not SS)			

3.1.3. Conclusion on lines of evidence for the EAS modalities

The study results summarized above indicate that isoflucypram does not act through the E, A, or S modalities in any experimental species tested.

The available data show no E-, A-, or S-mediated adversity and the ED criteria are thus not met for these modalities.

Therefore, isoflucypram is not an endocrine disruptor with regard to E, A, or S modalities.

3.2. Data sufficiency

The studies described in section 3.1.1 above include:

- Rat, mouse, and dog 28- and 90-day studies (90-day studies: OECD 408)
- Dog one-year study (OECD 452)
- Rat 2-year and mouse 18-month studies (OECD 453)
- Rat 2-generation reproduction study (OECD 416)
- Weanling Hershberger and uterotrophic screens (incorporating elements of the OECD 440 and 441 guidelines respectively)

Weights and macro- and microscopic appearance of organs sensitive to E, A, and S modalities were examined where appropriate in these studies, and no effects were observed which would indicate an adverse effect.

Thus, the data available is sufficient to conclude that isoflucypram is not an endocrine disruptor via the E, A, or S modalities.

4. Integrated lines of evidence for adverse effects and endocrine activity of isoflucypram (criterion 1 and 2) related to the T modality

4.1. Assessment of lines of evidence for the T modality

Guideline and mechanistic studies conducted in the relevant laboratory species have been conducted and assessed with regard to the effects of isoflucypram on endocrine homeostasis. The key information from these studies has been extracted and will be provided in two phases, in summary format in this document and then later detailed data tables will be submitted. This summary considers the weight of the available evidence to assess the potential for endocrine activity and adversity to determine if the criteria are met for endocrine disruption.

Original data can be found in the study reports listed in Table 3 as submitted in support of the isoflucypram application for approval under Regulation (EU) No 1107/2009.

Table 5: Studies containing data addressing the T modality.

Species	Duration / type	Reference
Rat	28 days	[redacted]2017;
	90 days	[redacted]2017;
	2 years	[redacted]2018;
	2-generation reproduction	[redacted]2018;
Mouse	28 days	[redacted] 2012;
	90 days	[redacted]2013;
	18 months	[redacted] 2017;
Dog	28 days	[redacted] 2014;
	90 days	[redacted] 2015;
	One year	[redacted]2017;

In the rat 28-day study, absolute thyroid weight was not statistically significantly increased in either males or females, although thyroid weight as a percentage of body weight was significantly increased in males at the top dose. Minimal to slight follicular cell hypertrophy was increased in both males and females at only the top dose.

In the 90-day study, relative thyroid weight was increased in males at only the top dose, with no effect on either absolute or relative thyroid weight in females. However, in both sexes there was an increase at the top dose in the incidence of both thyroid follicular cell hypertrophy and colloid alteration.

In the rat chronic study, absolute and / or relative liver weight was increased only in females, and only at the top dose, at both the 12-month and 24-month timepoints. There was no effect on liver weight, but the incidence of thyroid colloid alteration and follicular cell pigmentation was increased in both sexes at the top dose.

There were no effects on thyroid weight in the dog studies, nor were any histopathological findings observed in the thyroid in either the mouse or the dog.

4.2. Mode of action (MoA) analysis (criterion 3)

4.2.1. Introduction

Identification of isoflucypram as an inducer of hepatic Phase I and Phase II enzymes in the rat oriented the examination of potential modes of action for the minor effects observed in the thyroid. The mode-of-action studies included in this analysis are listed in Table 4.

Table 6: Studies containing data addressing the thyroid mode of action.

Species	Duration	Reference
Rat	7 days	[redacted]2018;
	28 days	[redacted]2013;
	28 days	[redacted]2018;

4.2.2. Evaluation of the interference of isoflucypram with thyroid hormone homeostasis in mammalian laboratory species

The concentrations of T3, T4, or TSH were not measured in standard guideline studies. In the 7-day and 28-day mechanistic studies, the concentrations of thyroid hormones were measured in female rats.

In the 7-day study, there was no biologically significant change in the concentration of either T3 or T4 at any dietary concentration of isoflucypram. The concentration of T3 was statistically significantly increased in two of the treated groups, but as there was no relationship to dose this observation is considered to be not related to treatment. The concentration of TSH was slightly but not statistically significantly increased in a number of groups, although there was no relationship to dose observed and thus the biological significance of this observation is unclear.

In the 28-day study which looked at hormone concentrations ([redacted]2013), there was similarly no change in the concentrations of either T3 or T4 across a range of tested concentrations. However, the concentration of TSH increased in a dose-related manner and was statistically significantly increased at the top dose tested.

Thus, the dietary administration of isoflucypram increased the concentration of TSH after 4 weeks of administration, although there was no alteration in the concentration of either T3 or T4.

4.2.3 Evidence for a non-specific, secondary mechanism of isoflucypram in the rat

As a CAR/PXR activator, isoflucypram was expected to induce hepatic Phase I and Phase II enzymes and to have a secondary effect on the thyroid in the rat. Enzyme activity was therefore measured in the 28-day rat study to determine whether this mode of action was present.

Isoflucypram caused a dose-related increase after 28 days in both absolute and relative liver weight in female rats, and increased relative liver weight in male rats at only the top dose. This was accompanied by an increase in hepatocellular hypertrophy at the same dose levels. Total hepatic cytochrome P450 concentration as well as the activities of EROD and PROD were statistically significantly increased in males, but not in females, from the mid dose. The activities of BROD and T4-UDPGT were significantly increased in both males and females from the mid dose. The extent of EROD induction in males was similar to the increase in total cytochrome P450 content, while increases of PROD and BROD were much greater, suggesting that the effects on EROD are linked more to an overall increase in cytochrome P450 isozymes and to a slight upregulation by either the CAR or the PXR of EROD activity rather than to an activation by isoflucypram of the AhR.

Absolute thyroid weight was not statistically significantly increased in either males or females, although thyroid weight as a percentage of body weight was significantly increased in males at the top dose. Minimal to slight follicular cell hypertrophy was increased in both males and females at only the top dose.

The following summary reflects the weight of the evidence assessment so far regarding liver and thyroid effects observed in guideline studies conducted with isoflucypram:

- Liver weight, histopathology, and (in the 28-day study) induction of specific Phase I and Phase II enzymes are all increased in a dose-related manner in the rat.
- Thyroid weight and / or histopathology are increased at doses at or above those which elicit liver effects in the liver.
- No effect on the thyroid is seen in the mouse or dog, even though liver weight and histopathology were increased in the mouse and dog as well as in the rat.
- The thyroid effects are thus limited to the rat, which is known to be uniquely sensitive to the secondary effects on the thyroid of CAR/PXR activation.

4.2.4. Human relevance

Regarding the physiology of the thyroid system, considerable differences exist between humans and other mammalian species, as shown below:

Table 7: Selected parameters of the thyroid system in humans, dogs and rats

Parameter	Human	Dog	Rat
Half-life of T4	5-9 days	8-16 h	0.5-1 day
Half-life of T3	1 day	5-6 h	0.25 day
Amount of T4 required in absence of functional thyroid gland	2.2 µg/kg bw/d	No data	20 µg/kg bw/d
T4 production (rate/kg bw)	1 x	No data	10 x
Primary T4 serum binding protein	TBG	No data	Albumin
High affinity T4 binding globulin (TBG) levels	High	Low (15% of human value)	Very low

Choksi et al. (2003), Daminet & Ferguson (2003), Jahnke et al. (2004).

TBG: thyroxine-binding globulin

A major difference between humans and experimental species such as the rat or dog is the much longer half-life of thyroid hormones; this longer half-life is considered to be due to strong binding of the thyroid hormones to thyroid binding globulin (TBG). This is a high-affinity binding protein, which is present at lower or even much lower (rat) concentrations in experimental animals compared to humans. High-affinity finding of thyroid hormones to carrier proteins such as TBG is considered to protect thyroid hormones against catabolism. In addition, glucuronidation of T4 by T4-UDPGT is only a minor route of hepatic T4 metabolism compared to rats (Richardson et al., 2014). Accordingly, in the light of considerable differences in thyroid physiology, the liver-mediated effects on the thyroid in rat studies with isoflucypram are not considered as relevant to humans.

4.2.5. Consideration of other mechanisms

The mechanistic studies described above examined the induction of specific cytochrome P450 isozymes known to be linked to activation of the CAR and / or PXR. They did not examine the possibility that isoflucypram acts through other modes of action on the rat thyroid. Alternative modes of action are presented in Table 6 and discussed in more detail following the table.

Table 8: Modes of action which can decrease thyroid hormone concentrations in rats.

MoA	Molecular Target	Positive reference compounds (examples)
Direct thyroid toxicity	Thyroid follicular epithelial cell or follicle	Pyrazole
Decreased release of thyroid hormones from the thyroid	Thyroglobulin degrading proteo-lytic enzymes	Lithium, iodide (excess)
Inhibition of iodide uptake into the thyroid	Sodium iodide symporter (NIS)	Perchlorate, thiocyanate Ouabain (indirectly)
Suppression of thyroid hormone synthesis	Thyroid peroxidase (TPO)	Sulfonamides and other aniline derivatives (aromatic amines) Propylthiouracil (thiourea derivative)
Displacement of thyroid hormones from binding sites in blood	T4-binding globulin (TBG) Transthyretin (TTR, T4-binding prealbumin) Albumin (ALB)	TBG: Salicylic acid derivatives Anthranilic acid derivatives Halogenated phenols Hydroxylated halogenated diphenylethers TTR: Salicylic acid derivatives Anthranilic acid derivatives Halogenated phenols Halogenated bisphenols Hydroxylated halogenated biphenyls Hydroxylated halogenated diphenylethers ALB: Salicylic acid derivatives Anthranilic acid derivatives Halogenated phenols
T3-Receptor-β2 agonism	T3-Receptor-β2 of pituitary and hypothalamus	Thyromimetics

Direct thyroid toxicity

Very few organic compounds have been identified that are directly toxic to the thyroid epithelial cell or the follicle. The decrease of thyroid hormone synthesis is the consequence of necrosis of the epithelial cells and/or rupture of the follicle (Szabo et al., 1978). Accordingly, direct toxicity to the thyroid results in considerable changes in thyroid histology.

As only minor histological changes were observed in regulatory studies with isoflucypram in the thyroid and these observations did not include either follicle rupture or epithelial cell necrosis, a direct effect of isoflucypram on the thyroid can be excluded.

Decreased release of thyroid hormones from the thyroid

Only few compounds, e.g. lithium or excess iodide, are known to decrease the release of thyroid hormones from the thyroid gland. The underlying mechanism is the inhibition of proteolytic enzymes degrading thyroglobulin in the follicular cell. A prominent feature of decreased thyroid hormone release is an increase in colloid accumulation (Kanno et al., 1994). Such changes in the colloid can be detected in the course of the histological examination of the thyroid.

Although colloid alteration was observed in the terminal-sacrifice animals in the rat 2-year study, this is not colloid accumulation and is not indicative of an inhibition of thyroid hormone release.

Inhibition of iodide uptake into the thyroid

The sodium iodide symporter (NIS) is important for the transport of iodide from the blood stream into the thyroid gland. The most well-known inhibitors of NIS are the so-called complex ions, ions having charge and radius comparable to iodide, e.g. perchlorate, thiocyanate and others. In this context it is important to know that thiocyanate may also be formed in the metabolism of cyanide. As NIS co-operates with Na/K-ATPase, also inhibitors of Na/K-ATPase such as the cardioactive/cardiotoxic ouabain can interfere with iodide uptake (Jones et al. 1996).

Isoflucypram is an organic compound and not able to release the mentioned ions or cyanide, and has no structural features which resemble ouabain.

Rat studies, especially 4 week studies, are considered as a very suitable study design to detect effects on thyroid function irrespective of the underlying mechanism. Perchlorate, administered via the drinking water (100 mg/L, corresponding to 13.6 to 27.8 mg/kg bw/day depending on water intake and body weight) to male rats over 2 weeks already decreased T3 and T4 to undetectable levels and increased TSH concentrations (Cunha & van Ravenzwaay, 2005 and references therein).

Accordingly, in the absence of any structural similarity to known NIS inhibitors and a clear correlation of thyroid-related effects with induction of hepatic UDP-glucuronyltransferase activitiy in the 4-week study, inhibition of NIS by isoflucypram is unlikely.

Suppression of TPO-catalyzed thyroid hormone synthesis

Numerous compounds have been reported to decrease thyroid hormone synthesis in vivo in the rat via interaction with TPO. A characteristic feature of these compounds is their effect on thyroid histology (hyperplasia, in the long run possibly adenoma and carcinoma) which is considered to be the consequence of a sustained counter-regulatory increase of TSH levels in response to impaired hormone synthesis. This has been reported for classical TPO inhibitors, namely sulfonamides (Astwood et al., 1943; Takayama et al., 1986), industrially used aromatic amines with structural features of thyroid hormones (e.g., phenylether, phenylthioether or diphenylmethane derivatives, Weisburger, 1983; Weisburger et al., 1984, Freyberger, 1994), and many, but not all thiourea derivatives (Astwood, 1943; Mellert et al., 2003). In a 4-week validation study for OECD on propylthiouracil (PTU), Mellert et al. (2003) have worked out the sensitivity of the various methodologies applied. Histopathological investigation of the thyroid was most sensitive to detect anti-thyroid effects of PTU (at ≥0.1 mg/kg bw/d), whereas decreases of T4 and increases of TSH levels were only detectable at ≥1 mg/kg bw/d (Mellert et al., 2003), and a decrease of T3 only occurred at 10 mg/kg bw/d.

Accordingly, regarding a) the high sensitivity of the 4-week rat study (Cunha & van Ravenzwaay, 2005) to detect interferences with thyroid hormone homeostasis in general and the known strong histopathological response of rat thyroid following TPO inhibition, and b) a clear plausible explanation for the modest hormonal changes in the 4-week rat study in females (liver enzyme induction) indicate that inhibition by isoflucypram of TPO-catalyzed thyroid hormone synthesis is unlikely.

Displacement of thyroid hormones from binding sites in blood

In the blood stream, thyroid hormones are bound to several proteins. Especially in humans thyroid hormones are bound to a very high extent (T4 to approx. 99.95%, T3 to approx. 99.5%). Binding proteins include (in the order of decreasing binding affinity in humans) T4-binding globulin (TBG), transthyretin (TTR, T4-binding prealbumin) and albumin. In adult rats TBG is of little relevance due to its very low concentration. A number of chemical classes have been identified to be able to displace thyroid hormones from TBG, such as simple aromatic compounds like salicylic and anthranilic acid derivatives and halogenated phenols and more complex compounds like hydroxylated halogenated diphenylether derivatives and perfluorinated alkyl acids. Displacers from TTR include salicylate and derivatives, anthranilic acid derivatives, phthalic acid (mono) esters and halogenated phenols as well as halogenated bisphenols and hydroxylated polychlorinated biphenyls, polychlorinated dibenzo-dioxins/furans and halogenated diphenylether derivatives and perfluorinated alkyl acids and sulfones. Reported displacers from albumin are salicylate and derivatives, anthranilic acid derivatives and halogenated phenols.

Isoflucypram has no structural similarities to any of the structural elements listed above which are associated with displacement of T4 from carrier protein binding sites. Displacement of thyroid hormones from binding sites occurs rapidly after exposure to a displacer (Brouwer, 1989) resulting in a strong decrease of total T4 levels, as the

released unbound T4 is rapidly metabolized. There was no effect on T4 concentrations after 4 weeks dietary administration of isoflucypram, which is not coherent with this proposed mode of action.

Thus, displacement of thyroid hormones from binding sites following treatment with isoflucypram is unlikely.

T3-Receptor-β2 agonism

T3-Receptor-β2 triggers the negative feedback of thyroid hormones in the hypothalamus and the pituitary (Williams, 2013). Agonism at this receptor would trigger an increased negative feedback with both decreased TSH and decreased thyroid hormone levels and a less active state of the thyroid.

Such effects have not been observed in studies with isoflucypram; TSH was increased in female rats after short-term administration, while T3 and T4 concentrations were unchanged. Furthermore, as a specific T3 receptor-β2 agonism may be considered unlikely, other agonistic responses mediated through other subtypes such as TRα1 (in brain, heart, skeleton and gastro-intestinal tract), and TRβ1 (in hypothalamus, pituitary, liver and lung) or both subtypes (in skeletal muscle and adipose tissue) would also be likely (see Williams, 2013). However, the available studies do not provide evidence for a thyromimetic effect (e.g., increased heart weight, changes in heart histology) of isoflucyrpam in experimental animals.

Accordingly, agonism of isoflucypram at T3-receptor-β2 or T3 receptors in general is unlikely.

4.2.6. Conclusions of the Mode of Action analysis

In repeat-dose studies conducted in experimental animals with isoflucypram, effects on the thyroid were only seen in the rat. Although thyroid weight is not measured in the mouse, thyroid histopathology was conducted in the 28- and 90-day and 18-month studies. The low number of animals in the dog studies may have obscured any effect on thyroid weight, however as for the mouse there was no effect on histopathology in either the 28- or 90-day studies or in the one-year study.

The available mechanistic data show that isoflucypram induces Phase I and Phase II liver enzymes known to be regulated by the CAR and / or PXR. Although other modes of action have not been experimentally disproven, the lack of coherence between the data with isoflucypram in the rat and the effects from other modes of action on thyroid function strongly suggests that secondary, indirect action of isoflucypram on the thyroid is responsible for the few effects shown.

The data available support the involvement of the CAR/PXR mode of action in the thyroid effects observed in the rat, through the following key points:

- An induction of CAR / PXR-dependent Phase I and Phase II enzymes in the liver – indication of the molecular initiating event
- Observation of thyroid changes at or above the dose level at which effects in the liver were observed – indication of dose dependency
- Observation of greater magnitude of thyroid changes with increasing study duration – indication of time dependency

The slight effects on thyroid histopathology and on TSH concentration in the rat can therefore be concluded to be secondary to the effects of isoflucypram on the liver, and to be not relevant for humans.

4.3. Data sufficiency

Thyroid hormone concentrations were measured in short-term mechanistic studies, and thyroid weight was measured in the subacute, subchronic, and long-term rat and dog studies, while thyroid histopathology was performed in the rat, mouse, and dog in the subacute, subchronic, and long-term studies. T-mediated parameters can therefore be considered sufficiently investigated.

As there was a change in concentration of TSH, a Mode of Action analysis must be conducted to address the potential for thyroid disruption in humans.

There is convincing evidence for a liver-mediated effect triggering an increase in TSH in the rat, including induction of CAR/PXR-dependent liver enzymes including UDPGT-T4, increased liver weight, and centrilobular hepatocellular hypertrophy.

Other modes of action which could affect the thyroid in experimental animals have been considered. However, in the absence of any thyroid effect in either the mouse or the dog, and in light of a lack of coherence between the few findings observed in the rat and the other modes of action, there is no convincing evidence for the involvement of any mode of action other than induction of liver enzymes leading to increased clearance of T4.

Based on the ED criteria, non-specific secondary consequences of other toxic effects are not considered to identify a substance as an endocrine disruptor. It can therefore be concluded that for the T modality, the ED criteria are not met for isoflucypram.

5. Conclusion

The data on isoflucypram has been assessed for adverse effects and endocrine activity for the E, A, and S modalities and for the T modality, as proposed in the recent ED guidance. It can be concluded that there is sufficient information available to be confident that isoflucypram is not an endocrine disruptor.

In guideline studies with isoflucypram, the liver was identified as the primary target organ, with effects also observed on the thyroid in rats only.

No reproductive effects were observed in either the F0 or the F1 animals, nor was there any treatment-related effect on sexual maturation in either males or females in the 2-generation reproduction study or the screening studies in immature male or female rats. There were no morphological or functional changes observed in any of the organs sensitive to the E, A, or S modalities, and no adverse effects were observed in any of these organs in any study conducted with isoflucypram. The E, A, and S modalities can be considered to have been sufficiently investigated, and it can be concluded that the ED criteria are not met for these modalities.

Thyroid gland weight was increased slightly in high-dose groups in the 28-day and 90-day studies, with some follicular cell hypertrophy and colloid alteration observed at the top doses. In the 2-year study, thyroid weight was increased and some increase in the incidence of thyroid colloid alteration and follicular cell pigmentation were observed in females only. There was no effect on T3 or T4 concentration after short-term administration, but TSH was increased.

Mode of action studies in the female rat showed that isoflucypram induces liver enzymes which catabolize T4. In light of considerable differences in thyroid physiology between the rat and humans, the liver-mediated effects of isoflucypram on the thyroid is not considered relevant for humans. As the effects on the thyroid are secondary to the effects of isoflucypram on the liver, it can be concluded that isoflucypram shows no adversity with regard to the T modality.

In the absence of any effects on E, A, or S modalities, and in the absence of any adversity in the T modality, the ED criteria are not met for isoflucypram.

References

Astwood EB. The chemical nature of compounds which inhibit the function of the thyroid gland. J Pharmacol 1943; 78: 79-89

Astwood EB, Sullivan J, Bissell A, Tyslowitz. Action of certain sulfonamides and of thiourea upon the function of the thyroid gland of the rat. Endocrinology 1943; 32: 210-225

Brouwer A. Inhibition of Thyroid Hormone Transport in Plasma of Rats by Polychlorinated Biphenyls. Arch Toxicol 1989; Suppl 13: 440-445

Choksi NY, Jahnke GD, St Hilaire C, Shelby M. (2003). Role of thyroid hormones in human and laboratory animal reproductive health. Birth Defects Res B 2003; 68:479–491.

Cunha GC-P, van Ravenzwaay B. Evaluation of mechanisms inducing thyroid toxicity and the ability of the enhanced OECD Test Guideline 407 to detect these changes. Arch Toxicol 2005; 79: 390–405

Daminet S, Ferguson DC. Influence of drugs on thyroid function in dogs. J Vet Intern Med 2003; 17: 463-472

Freyberger A. Irreversible inhibition of thyroid peroxidase (TPO) by thyreotoxic aromatic amines. Naunyn-Schmiedeberg's Arch Pharmacol 1994; 349 Suppl: R 110

Jahnke GD, Choksi NY, Moore JA, Shelby MD. Thyroid toxicants: assessing reproductive health effects. Environ Health Perspect 2004; 112: 363–368.

Jones PA, Pendlington RU, Earl LK, Sharma RK, Barratt MD. In vitro investigation of the direct effects of complex anions on thyroidal iodide uptake: indentification of novel inhibitors. Toxicol In Vitro 1996; 10: 149-160

Kanno J, Nemoto T, Kasuga T, Hayashi Y. Effects of a six-week exposure to excess iodide on thyroid glands of growing and nongrowing male Fischer-344 rats. Toxicol Pathol 1994; 22, 23-28

Mellert W, Deckardt K, Walter J, Gfatter S, van Ravenzwaay B. Detection of endocrine-modulating effects of the antithyroid acting drug 6-propyl-2-thiouracil in rats, based on the ''Enhanced OECD Test Guideline 407.'' Regul Toxicol Pharmacol 2003; 38: 368–377

Richardson VM, Ferguson SS, Sey YM, DeVito MJ. In vitro metabolism of thyroxine by rat and human hepatocytes. Xenobiotica 2014; 44: 391-403.

Szabo S, Horvath E, Kovacs K, Larsen PR. Pyrazole-induced thyroid necrosis: a distinct organ lesion. Science 1978; 199: 1209-1210

Takayama S, Aihara K, Onodera T, Akimoto T. Antithyroid effects of propylthiouracil and sulfamonomethoxine in rats and monkeys. Toxicol Appl Pharmacol 1986; 82: 191-199

Williams GR. Physiologically distinct roles for thyroid hormone receptor isoforms. Endocrine Abstracts 2013; 31 S7.1

Weisburger EK, Murthy ASK, Lilja HS, Lamb IV JC. Neoplastic responses of F344 rats and B6C3F1 mice to the polymer and dyestuff intermediates 4,4'-methylenebis(N,N-dimethyl)-benzenamine, 4,4' oxydianiline, and 4,4'-mehylenedianiline. J Nat Cancer Inst 1984; 72: 1457-1463

Weisburger EK. Species differences in response to aromatic amines. In: Langenbach R, Nesnow S, Rice JM (Eds) Organ and Species Specifity in Chemical Carcinogenesis. Plenum Press, New York, 1983