Committee for Risk Assessment

RAC

Opinion

proposing harmonised classification and labelling
at EU level of

Reaction mass of 3-(difluoromethyl)-1-methyl-*N*-[(1*RS*,4*SR*,9*RS*)-1,2,3,4-tetrahydro-9-isopropyl-1,4-methanonaphthalen-5-yl]pyrazole-4-carboxamide and 3-(difluoromethyl)-1-methyl-*N*-[(1*RS*,4*SR*,9*SR*)-1,2,3,4-tetrahydro-9-isopropyl-1,4-methanonaphthalen-5-yl]pyrazole-4-carboxamide [≥78% *syn* isomers ≤15% *anti* isomers relative content]; isopyrazam

EC Number: -
CAS Number: 881685-58-1

CLH-O-0000006915-65-01/F

Adopted

10 December 2020

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CLH-O-0000006915-65-01/F

OPINION OF THE COMMITTEE FOR RISK ASSESSMENT ON A DOSSIER PROPOSING HARMONISED CLASSIFICATION AND LABELLING AT EU LEVEL

In accordance with Article 37 (4) of Regulation (EC) No 1272/2008, the Classification, Labelling and Packaging (CLP) Regulation, the Committee for Risk Assessment (RAC) has adopted an opinion on the proposal for harmonised classification and labelling (CLH) of:

Chemical name: **Reaction mass of 3-(difluoromethyl)-1-methyl-*N*-[(1*RS*,4*SR*,9*RS*)-1,2,3,4-tetrahydro-9-isopropyl-1,4-methanonaphthalen-5-yl]pyrazole-4-carboxamide and 3-(difluoromethyl)-1-methyl-*N*-[(1*RS*,4*SR*,9*SR*)-1,2,3,4-tetrahydro-9-isopropyl-1,4-methanonaphthalen-5-yl]pyrazole-4-carboxamide [≥78% *syn* isomers ≤15% *anti* isomers relative content]; isopyrazam**

EC Number: **-**

CAS Number: **881685-58-1**

The proposal was submitted by the **United Kingdom (taken over by Norway)** and received by RAC on **1 August 2019.**

In this opinion, all classification and labelling elements are given in accordance with the CLP Regulation.

PROCESS FOR ADOPTION OF THE OPINION

The United Kingdom (taken over by Norway) has submitted a CLH dossier containing a proposal together with the justification and background information documented in a CLH report. The CLH report was made publicly available in accordance with the requirements of the CLP Regulation at ***http://echa.europa.eu/harmonised-classification-and-labelling-consultation/*** on **23 September 2019**. Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by **22 November 2019**.

ADOPTION OF THE OPINION OF RAC

Rapporteur, appointed by RAC: **Miguel A. Sogorb** supported by **Ruth Moeller**

Co-Rapporteur, appointed by RAC: **Raili Moldov**

The opinion takes into account the comments provided by MSCAs and concerned parties in accordance with Article 37(4) of the CLP Regulation and the comments received are compiled in Annex 2.

The RAC opinion on the proposed harmonised classification and labelling was adopted on **10 December 2020** by **consensus**.

Classification and labelling in accordance with the CLP Regulation (Regulation (EC) 1272/2008)

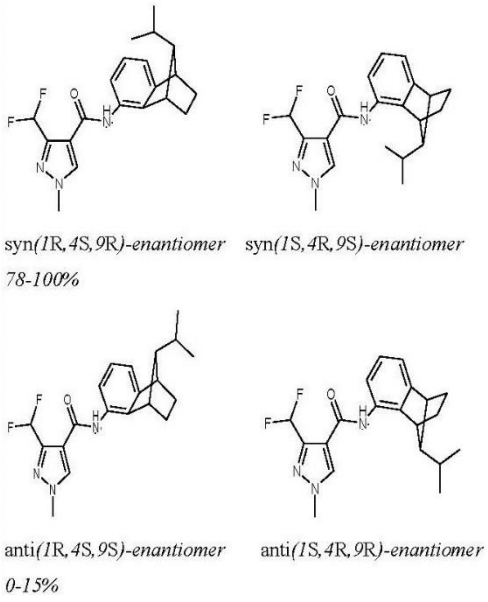
	Index No	Chemical name	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors and ATE	Notes
					Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)	Suppl. Hazard statement Code(s)		
Current Annex VI entry	No current Annex VI entry										
Dossier submitters proposal	TBD	Reaction mass of 3-(difluoromethyl)-1-methyl-N-[(1RS,4SR,9RS)-1,2,3,4-tetrahydro-9-isopropyl-1,4-methanonaphthalen-5-yl]pyrazole-4-carboxamide and 3-(difluoromethyl)-1-methyl-N-[(1RS,4SR,9SR)-1,2,3,4-tetrahydro-9-isopropyl-1,4-methanonaphthalen-5-yl]pyrazole-4-carboxamide [≥78% *syn* isomers ≤15% *anti* isomers relative content]; isopyrazam	-	881685-58-1	Repr. 1B Skin Sens. 1B Aquatic Acute 1 Aquatic Chronic 1	H360D H317 H400 H410	GHS07 GHS08 GHS09 Dgr	H360D H317 H410		M=10 M=10	
RAC opinion	TBD	Ditto; isopyrazam	-	881685-58-1	Carc. 2 Repr. 1B Skin Sens. 1B Aquatic Acute 1 Aquatic Chronic 1	H351 H360D H317 H400 H410	GHS07 GHS08 GHS09 Dgr	H351 H360D H317 H410		Repr. 1B; H360D: C ≥ 3 % M=10 M=10	
Resulting Annex VI entry if agreed by COM	TBD	Ditto; isopyrazam	-	881685-58-1	Carc. 2 Repr. 1B Skin Sens. 1B Aquatic Acute 1 Aquatic Chronic 1	H351 H360D H317 H400 H410	GHS07 GHS08 GHS09 Dgr	H351 H360D H317 H410		Repr. 1B; H360D: C ≥ 3 % M=10 M=10	

GROUNDS FOR ADOPTION OF THE OPINION

RAC general comment

Isopyrazam is an active substance in the scope of Regulation (EC) 1107/2009 and is used as a broad-spectrum foliar fungicide. Isopyrazam is not listed in Annex VI of CLP and has not been considered for harmonised classification and labelling previously. Isopyrazam is not registered under REACH.

The substance identified by the provisional ISO name 'isopyrazam' (a reaction mass of 3-(difluoromethyl)-1-methyl-N-[(1RS,4SR,9RS)-1,2,3,4-tetrahydro-9-isopropyl-1,4-methanonaphthalen-5-yl]pyrazole-4-carboxamide and 3-(difluoromethyl)-1-methyl-*N*-[(1RS,4SR,9SR)-1,2,3,4-tetrahydro-9-isopropyl-1,4-methanonaphthalen-5-yl]pyrazole-4-carboxamide [>78% *syn* isomers <15% *anti* isomers relative content]) is a multi-constituent substance containing two diasteroisomers (epimers) designated *syn* and *anti* isomers (see figure below). In some test reports they are referred as SYN534969 and SYN534968 respectively. Both isomers are biologically active and, according to the Commissioning Implementing Regulation (EC) No 1037/2012 approving the active substance 'isopyrazam', the approved specification contains the *syn* and *anti* isomers at a percentage ranging from 78-100% *syn* and 0-15% *anti*, with an overall minimum purity of 92%.



Structural formula of isopyrazam

Most of the toxicology studies have been conducted on batches with an overall purity of 96.4% which consists of 92.8% *syn* and 7.2% *anti* (referred to as 93:7 *syn*:*anti* in this report). Additional studies are available with batches containing 90:10, 69.7:30.3 (referred to as 70:30 in this report) and 50:50 *syn*:*anti* isomers, along with a number of studies on the pure *syn* and *anti* isomers themselves. These data are included as supporting information. The environmental studies have been conducted on batches with varying concentrations of the *syn* and *anti* isomers and this is specified in the relevant sections.

One MSCA raised an identification issue for the substance as regard the maximal content of the *anti* isomer. The Dossier Submitter (DS) clarified that isopyrazam specifications have been developed to contain a maximum of 15% *anti* isomer.

RAC evaluation of physical hazards

Summary of the Dossier Submitter's proposal

Explosive

In a standard explosivity study (EEC, A.14) there was no evidence of shock, friction or thermal sensitivity and isopyrazam was not considered to be explosive within the criteria of this study.

Flammable solid

In the preliminary study (EEC, A.10), the substance melted on contact with the flame but did not burn. The full study was not conducted. The criteria for classification as a flammable solid are not met.

Self-reactive substance or mixture

No signs of exothermic decomposition were observed in a study conducted in accordance with OECD TG 113 at temperatures up to 200˚C. In the OECD TG 103 studies conducted with the *syn* and *anti* isomers, exothermic decomposition was noted from 261˚C (with 192 J/g) and 274˚C (with 143 J/g) respectively. The available data indicate that the substance does not warrant classification as self-reactive.

Pyrophoric solid

No specific studies are available. However, the available studies report the use of isopyrazam without any special conditions regarding the exclusion of air or moisture and no incidences of self-ignition have been reported. Hence classification is not warranted.

Self-heating substance or mixture

A study conducted in accordance with EEC A.16 is available. In this study, isopyrazam did not self-ignite up to a temperature of 400 ˚C. This study is not directly comparable with the CLP criteria and no further data were available.

Substance or mixture which in contact with water emits flammable gas

No data derived in accordance with the test method recommended by CLP have been provided. However, isopyrazam has been handled in water within many of the studies available in the draft assessment report, and there are no reports of violent reaction and emission of gas. Hence classification is not waranted.

Oxidising solid

In a provided study (EEC, A.17), the maximum burning rate of the test substance/cellulose mixture was determined to be 1.5 mm/s compared to 2.8 mm/s for the reference substance (barium nitrate)/cellulose mixture. However, these results are not directly comparable with the CLP criteria. It should be also noted that whilst the substance contains oxygen and fluorine, these elements are only bound to carbon. Weighing these factors, the substance is not considered to be an oxidising solid.

Substance or mixture corrosive to metals

No data available. Test C.1 in the UN RTDG Manual of Tests and Criteria is intended to determine the corrosive properties of liquids and solids that may become liquid during transport. Isopyrazam is a solid, with a melting point higher than 55˚C (estimated > 130˚C) and with low water solubility (1.05 and 0.55 mg/L for the *syn* and *anti* isomers respectively). Furthermore,

based on experience in manufacture and handling, the substance does not materially damage metallic containers. Classification as corrosive to metals is not warranted.

Comments received during consultation

One MSCA pointed out a mistake in referencing a value of solubility of the substance in dichloromethane, this was corrected by the dossier submitter.

Assessment and comparison with the classification criteria

Explosive

The DS referred to the results of a standard explosivity study indicating no evidence of shock, friction or thermal sensitivity leading to inconclusive data for classification. RAC notes that the exothermic decomposition energy is less than 500 J/g and the onset of exothermic decomposition is below 500°C, hence no classification is warranted.

Flammable solid

The presented preliminary study results showed the substance melted on contact with flame but did not burn. As preliminary test according to A.10 (identical to a preliminary test according to UN N.1) was negative, RAC agrees with the DS that the data is conclusive but not sufficient for classification.

Self-reactive substance or mixture

Although no exothermic decomposition occurred in an OECD 113 test with temperatures up to 200°C, it did occur at higher temperatures (*syn*: 261°C; *anti*: 274°C) in an OECD 103 test. Nevertheless, the heat of decomposition was less than 300 J/g, and therefore no classification is warranted based on conclusive information (CLP, Annex I, 2.8.2.1).

Pyrophoric solid

No specific studies are available. However, isopyrazam has been handled in air in the available studies and no incidences of self-ignition have been reported.

Experience in manufacture and handling shows that the substance does not ignite spontaneously on coming into contact with air at normal temperatures (i.e. the substance is known to be stable based on reports of the available studies). RAC supports the DS proposal for no classification.

Self-heating substance or mixture

As the study conducted in accordance with EEC A.16 is not sufficient to conclude on the classification and no further data is available, RAC proposes not to classify the substance in this hazard class due to inconclusive data.

Substance or mixture which in contact with water emits flammable gas

As most of the studies with isopyrazam are performed in aqueous solutions and there are no reports for emission of gas, RAC support DS proposal for no classification.

Oxidising solid

As the substance contains oxygen and fluorine and these elements are chemically bonded only to carbon, RAC agrees with the DS proposal for no classification.

Substances or mixtures corrosive to metals

RAC agrees with the DS that the available data indicating isopyrazam being a solid with a melting point > 130 °C and having low water solubility, that the substance can be considered not classifiable as corrosive to metals.

Conclusion

Overall, RAC agrees with the rational of the DS and that **no classification with regards to physical hazards is warranted for isopyrazam**.

HUMAN HEALTH HAZARD EVALUATION

RAC evaluation of acute toxicity

Summary of the Dossier Submitter's proposal

The DS assessed a database with three up-down tests (OECD 425) for acute oral toxicity (including pure isomers and three different *syn*:*anti* ratios), one OECD 402 test for assessing acute dermal toxicity and one OECD 403 test for assessing acute inhalation toxicity. All these studies were performed according to GLP and allowed the DS to propose no classification of isopyrazam for acute toxicity by any of the three relevant routes.

Comments received during consultation

One MSCA considered that it cannot be excluded that the LD_{50} could be less than 2000 mg/kg bw with a mixture of isopyrazam containing 15% of the *anti* isomer and therefore proposed Acute Tox 4 for oral route. The DS replied that considering the ATE of the pure *anti* isomer the ATE of the substance containing less than 15% could be calculated to be >2000 mg/kg bw.

A second MSCA also demanded Acute Tox 4 for oral route with a 70:30 *syn*:*anti* isomer ratio. DS agreed with the comment. However, RAC notes that the CLH report is covering a ratio of up to 15% of *anti* isomer.

Assessment and comparison with the classification criteria

Table 1 summarises the acute toxicity studies presented in the CLH report.

Table 1: Summary of animal studies on acute toxicity with isopyrazam.

Study	Dose level	Results	Reference
Up-and-down procedure Gavage OECD 425 GLP HanRcc:WIST rats 1 female per dose: 175, 275 and 2500 mg/kg bw	Isopyrazam (batch: SMU6AP001) Purity: 96.4% **93:7 *syn*:*anti*** Vehicle: 0.5% carboxymethylcellulose 2500 mg/kg bw (limit test) 2000, 275 and 175 mg/kg bw (main test)	<u>In all doses and all animals:</u> No unusual findings in necropsies and body weights within normal ranges <u>Limit test:</u> 1/1 at 2500 mg/kg bw killed *in extremis* (sedation, ruffled fur, hunched posture, poor coordination and recumbency) <u>Main test:</u> 175 mg/kg bw: 1/1 female survived with ruffled fur on day of dosing	Anonymous (2007a)

3 females for 2000 mg/kg bw	Observation period: 15 days	275 mg/kg bw: 1/1 female survived with ruffled fur and hunched posture on the day of dosing 2000 mg/kg bw: 3/3 females survived with hunched posture, poor condition, sedation on the day of dosing up to day 4 and ruffled fur to day 7 **Conclusion:** **LD_{50} >2000 mg/kg bw**	
Up-and-down procedure Gavage OECD 425 GLP HanRcc:WIST rats 175 mg/kg bw: 1 female 550 mg/kg be: 4 females 2000 mg/kg bw: 7 females	Isopyrazam (batch: SMU7DP017) Purity: 90.8% (w/w) **70:30 *syn*:*anti*** Vehicle: 0.5% carboxymethylcellulose 2000 mg/kg bw (limit test) 2000, 550 and 175 mg/kg bw (main test) Observation period: 15 days	<u>In all doses and all animals:</u> Body weights within normal ranges <u>Limit test:</u> 1/1 female at 2000 mg/kg bw was killed *in extremis* a few hours after dosing <u>Main test:</u> 175 mg/kg bw: 1/1 female survived with ruffled fur on day of dosing 550 mg/kg bw: 4/4 females survived with hunched posture, poor coordination, sedation on day of dosing and ruffled posture to day 4 2000 mg/kg bw: 5/7 females killed *in extremis*, 1 female killed 5 hours after dosing and 1 animal killed 2 days after dosing. Clinical signs: Sedation, poor coordination, hunched posture, ruffled fur, cold to touch, ventral or lateral recumbency and convulsions. 3/5 animals had distended stomach, liquid contents in the duodenum, grey material in the stomach/duodenum and an empty jejunum/ileum. 2/7 females survived with poor coordination, sedation on the day of dosing and for up to 3 days, ruffled fur for up to 7 days. **Conclusion:** **LD_{50} ≤ 2000 mg/kg bw (95% confidence interval 864-4210 mg/kg)**	Anonymous (2008a)
Up-and-down procedure, gavage OECD 425 GLP Anonymous (2006a) HanRcc:WIST rats <u>Pure *syn*</u>: 5 females for 2000 mg/kg bw	**Pure *syn* epimer** (SYN534969) (batch SMU6BP001) (purity: 99%)	<u>In all doses and all animals:</u> No unusual findings in necropsies and body weights within normal ranges <u>SYN534969 (pure *syn*)</u> 2000 mg/kg: 5/5 females survived with hunched posture, slight to moderate poor coordination, riffled fur on day of dosing and for up to 5 days **Conclusion:** **LD_{50} >2000 mg/kg bw** <u>SYN534968 (pure *anti*)</u>	Anonymous (2006a)

Pure *anti*: 3 females per dose for 175 and 550 mg/kg be and 1 female for 2000 mg/kg bw 50:50 *syn*:*anti*: 3 females per dose for 175 and 550 mg/kg be and 1 female for 2000 mg/kg bw	**Pure *anti* epimer** (SYN534968) (batch: SMU6BP001) (purity: 99.5%) Isopyrazam (batch: SMU6CP014) (purity: 98.2%) **50:50 *syn*:*anti* ratio:** 2000 mg/kg bw (pure *syn*) 2000, 550 and 175 mg/kg bw (pure *anti*) 2000, 550 and 175 mg/kg bw (50:50 *syn*:*anti*)	2000 mg/kg bw: 1/1 female killed *in extremis* 2 hours post-dose with ventral recumbency and marked poor coordination 550 mg/kg bw: 3/3 females killed *in extremis* 1-2 hours post-dose with hunched posture, desation and ventral recumbency 175 mg/kg bw: 3/3 females survived with huched posture, slight poor coordination and ruffled fur on day of dosing and for up to 3 days **Conclusion:** **LD_{50} = 310.2 mg/kg bw (95% confidence interval 175-550 mg/kg bw)** Isopyrazam (50:50 *syn*:*anti*) 2000 mg/kg bw: 1/1 female killed *in extremis* 2 hours post-dose with vertebral recumbency and sedation 550 mg/kg bw: 3/3 females killed *in extremis* 1-2 hours post-dose with vertebral recumbency and sedation 175 mg/kg bw survived: 3/3 females survived with vertebral recumbency on day of dosing, hunched posture, poor coordination, ruffled fur on day of dosing and for up to 3 days **Conclusion:** **LD50 = 310 mg/kg bw (95% confidence interval 175-550 mg/kg)**	
Acute dermal toxicity study OECD 402 GLP HanRcc:WIST rats 5 animals/sex	Isopyrazam (batch: SMU6AP001) Purity: 96.4 % (w/w) **93:7 *syn*:*anti*** Vehicle: Moistened with purified water 5000 mg/kg bw Application area: 16 cm2 Duration: 24 hours Observation period: 14 days	No deaths No signs of systemic toxicity or local effects No adverse macroscopic findings Conclusion: LD50 > 5000 mg/kg bw	Anonymous (2007b)
Acute inhalation toxicity study Nose-only exposure Dust	Isopyrazam (batch: SMU6AP001) Purity: 96.4% **93:7 *syn*:*anti***	All animals survived There were no treatment related signs of toxicity or adverse macroscopic findings **Conclusion:** **LC50 > 5.28 mg/l**	Anonymous (2006)

OECD 403 GLP HsdBrlHan Wistar rats 5 animals/sex	MMAD: 2.88±3.01 µm (1 hour) and 1.82±1.89 µm (3 hours) Mean concentration: 5.28±0.08 mg/l air Exposure duration: 4 hours Observation period: 15 days

The acute oral toxicity study on isopyrazam (with a 93:7 *syn*:*anti* ratio) gave an LD_{50} > 2000 mg/kg bw (Table 1). It is noted that, according to the approved product specification, isopyrazam can contain a maximum of 15% of the *anti* isomer and additional studies on the individual *syn* and *anti* isomers indicate that the *anti* isomer is more acutely toxic than the *syn* isomer. This is evident in further studies on material containing the *syn*:*anti* isomers in a ratio of 70:30 and 50:50, where the LD_{50} was found to be < 2000 mg/kg bw in each case. No data are available on isopyrazam containing 15% of the *anti* isomer.

The acute dermal LD_{50} of the 93:7 *syn*:*anti* specification of isopyrazam is >5000 mg/kg bw (Table 1). The approved specification of isopyrazam for use as an active substance in plant protection products allows for a content of the more acutely toxic (by the oral route) *anti* isomer at a maximum of 15%, which is greater than the 7% in the specification investigated for dermal toxicity in this study. However, as the LD_{50} of the specification tested for acute dermal toxicity was > 5000 mg/kg bw/d, a slight increase of the *anti* isomer would not be expected to result in an LD_{50} smaller than the 2000 mg/kg bw cut-off value for classification in accordance with CLP.

The acute inhalation toxicity of isopyrazam dust (93:7 *syn*:*anti* specification) was investigated in a nose-only exposure, inhalation toxicity study. The LC_{50} of isopyrazam (93:7 *syn*:*anti* specification) is > 5.28 mg/l (Table 1). A slight increase in the *anti* isomer to 15%, as in the approved isopyrazam specification for use as an active substance in plant protection products, would not be expected to substantially lower the LC_{50}. In the acute oral toxicity studies, the signs of toxicity observed in the animals killed *in extremis* that had received the specification of isopyrazam containing 70:30 *syn*:*anti* isomers were mainly related to the oral exposure route (gastrointestinal effects comprising a distended stomach, liquid contents in the duodenum, a grey material in the stomach/duodenum and an empty jejunum/ileum). Therefore, the acute toxicity of isopyrazam is not likely to be greater when administered via the inhalation route compared to the oral route.

Comparison with the criteria

According to the CLP criteria, classification for acute oral toxicity is warranted if the ATE (LD50) of a substance is ≤ 2000 mg/kg bw. The acute oral toxicity study on isopyrazam (with a 93:7 *syn*:*anti* ratio) gave an LD_{50} above this value. The database shows that the *anti* isomer is clearly more toxic than the *syn* isomer and RAC notes that no toxicity study is available with a *syn*:*anti* ratio 85:15; which should be more toxic isomeric ratio compared to 97:3 ratio. RAC notes that considering the available information the LD50 of the 85:15 *syn*:*anti* ratio could be higher than 2000 mg/kg bw. Nevertheless, some additional uncertainties, such as the ones caused by males not being tested (which could be a much more susceptible sex than females) were noted. Overall, RAC supports the DS proposal for **no classification of isopyrazam for acute oral toxicity** but in this case **based on inconclusive data.**

According to the CLP criteria, a substance is classified for acute dermal toxicity if the LD_{50} value is ≤ 2000 mg/kg bw. In the available study, isopyrazam (**93:7 *syn*:*anti***) was found to have an

LD_{50} value of > 5000 mg/kg bw. A slight increase in the *anti* isomer, which is more acutely toxic by the oral route, would not be expected to result in an LD_{50} lower than 2000 mg/kg bw. Therefore, RAC supports the DS's proposal for **no classification of isopyrazam for acute dermal toxicity.**

In accordance with the CLP criteria, a substance (dust and mist) is classified for acute inhalation toxicity if it has an LC_{50} value of ≤ 5 mg/l air. In the available study the LC_{50} value for isopyrazam (93:7 *syn*:*anti*) is > 5.28 mg/l air, tested in both sexes and without any signs of toxicity. RAC expects the acute inhalation toxicity effects of the *anti* isomer to be similar to the oral exposure route, i.e., a slight increase in the *anti* isomer, which is more acutely toxic by the oral route, would not be expected to result in an LC_{50} lower than 5 mg/l. RAC supports the DS's proposal for **no classification of isopyrazam for acute inhalation toxicity.**

RAC evaluation of specific target organ toxicity – single exposure (STOT SE)

Summary of the Dossier Submitter's proposal

The DS proposed no classification of isopyrazam based on the absence of specific effects reported in the acute toxicity tests (see Table 1) and the absence of neurotoxicity in one acute neurotoxicity study using doses up to 2000 mg/kg bw.

Comments received during consultation

One MSCA supported the DS's proposal for no classification of isopyrazam for STOT SE.

Assessment and comparison with the classification criteria

RAC notes the absence of organ-specific effects in the acute toxicity studies via oral, dermal and inhalation routes (Table 1). In these studies, at doses up to 2500 mg/kg bw, the observed clinical signs (ruffled fur, hunched posture, poor coordination, sedation and ventral recumbency) are commonly associated with general toxicity and not indicative of toxic effects on any specific target organ.

The CLH report presents also an acute neurotoxicity study (Anonymous 2009a) performed in rats conducted according to OECD 424 Guideline and observing GLP. On this study, 10 rats/sex/group were treated with a single dose of 30, 250 or 2000 mg/kg bw of isopyrazam (purity 96.4%, 93:7 *syn*:*anti*) in 0.5% w/v caroxymethylcellulose in water. Animals were further observed for 16 days. All animals survived until the end of the observation period. Body weight gain in females was 30% and 33% lower than controls in the 250 and 2000 mg/kg bw dose groups, respectively, during the first week of dosing. Observations noted at one-hour post-dose comprised decreased activity (250 and 2000 mg/kg bw; males and females), weak appearance (250 and 2000 mg/kg bw/d; females) and swaying gait (250 mg/kg bw; females). Reduced activity, along with reduced rearing activity was also noted during the Functional Observational Battery (FOB) assessments on day one only (250 and 2000 mg/kg bw; males and females); however, the incidence was low and no dose-response was evident. There was no effect on brain weight and no microscopic or macroscopic findings in tissues that would suggest a specific effect on the nervous system.

Comparison with the criteria

None of the single dose animal studies contained in the CLH report provided evidence of organ specific toxicity; which does not support classification as STOT SE 1 or 2. Moreover, no respiratory tract irritation was found in the studies; hence not supporting classification as STOT SE 3 H335. According to CLP classification criteria, narcotic effects observed in animal studies may include lethargy, lack of coordination, loss of righting reflex and ataxia, and are usually readily reversible on cessation of exposure with no permanent damage or changes. Poor coordination and sedation (lethargy) were observed in acute toxicity studies at doses below and above LD50 value. Decreased activity and swaying gait (may be considered as lethargy and poor coordination/ataxia) were detected one-hour post exposure in mid and high dose group during the acute neurotoxicity study. These effects may be indications of transient CNS depression. However, they may also be considered as indications of general or secondary toxicity. In the absence of any indications of potential neurotoxicity-based effects observed in other studies, or on the structure or mechanism/mode of action of the substance, and considering that the effects were observed still at substantially high doses, RAC does not consider that the criteria for classification regarding narcotic effects are met. Therefore, RAC supports the DS's proposal for **no classification of isopyrazam as STOT SE.**

RAC evaluation of skin corrosion/irritation

Summary of the Dossier Submitter's proposal

DS proposed no classification of isopyrazam for skin irritation based on the results of one guideline- and GLP compliant skin irritation study conducted in rabbits using the 93:7 *syn*:*anti* specification of isopyrazam where no erythema and no oedema was reported.

Comments received during consultation

One MSCA supported the DS's proposal for no classification of isopyrazam for skin irritation/corrosion.

Assessment and comparison with the classification criteria

Table 2 summarises the animal study on skin corrosion/irritation found in the CLH report.

Table 2: Summary of the animal study on skin corrosion/irritation with isopyrazam.

Study	Dose level	Results	Reference
Skin irritation study OECD 404 GLP New Zealand White rabbits 1 male and 2 females	Isopyrazam (batch: SMU6AP001) Purity: 96.4% **93:7 *syn*:*anti*** 0.5 g moistened with 0.5 ml purified water Exposure duration: 4 hours	Skin reactions were assessed at 1, 24, 48 and 72 hours post-exposure Mean scores for each animal (over 24, 48 and 72 hours): Erythema: 0, 0, 0 Oedema: 0, 0, 0 **Conclusion: Not a skin irritant**	Anonymous (2006b)

The skin irritating/corrosive potential of isopyrazam (93:7 *syn*:*anti*) has been investigated in rabbits (Table 2). There were no signs of irritation at any of these time points (mean scores for both erythema and oedema were 0). As no signs of irritation were observed in the 93:7 specification of isopyrazam, a slight increase in the content of the *anti* isomer, which is more acutely toxic by the oral route, would not be expected to increase the skin irritation potential of isopyrazam.

Comparison with the criteria

None of the criteria demanded for triggering classification are met for isopyrazam since the average scores for each animal (over 24, 48 and 72 hours) were 0, 0 and 0 for both erythema and oedema. Therefore, RAC supports the DS's proposal for **no classification of isopyrazam as skin irritant.**

RAC evaluation of serious eye damage/irritation

Summary of the Dossier Submitter's proposal

The eye irritating potential of isopyrazam (93:7 *syn*:*anti*) was investigated in rabbits. DS proposed no classification of isopyrazam for eye damage/irritation based on the results of one guideline and GLP compliant study where the mean score at 24, 48 and 72 hours were of 0 (cornea), 0 (iris), 1 (conjunctival redness) and 0-0.3 (conjunctival chemosis).

Comments received during consultation

One MSCA supported the DS's proposal for no classification of isopyrazam for serious eye/damage.

Assessment and comparison with the classification criteria

Table 3 summarises the animal study on serious eye damage/eye irritation found in the CLH report.

Table 3: Summary of the animal study on eye damage/irritation with isopyrazam.

Study	Dose level	Results	Reference
Eye irritation study OECD 405 GLP New Zealand White rabbits 1 male and 2 females	Isopyrazam (batch: SMU6AP001) Purity: 96.4% (w/w) ***Syn*:*anti* ratio: 93:7** 0.1 g	Eye reactions were scored 1, 24, 48 and 72 hours and 7 days after instillation There were no deaths or clinical signs of toxicity Mean scores at 24, 48 and 72 h: Cornea: 0, 0, 0 Iris: 0, 0, 0 Conjunctival redness: 1, 1, 1 Conjunctival chemosis: 0.3, 0, 0.3 All observed signs had fully reversed by day 7 of treatment	Anonymous (2007)

The potential of isopyrazam to irritate the eyes was investigated in one male and two female New Zealand White rabbits (Table 3). The mean scores for both the cornea and the iris were 0, 0 and 0 and the mean scores for conjunctiva redness and erythema were 1, 1, 1 and 0.3, 0, 0.3; respectively. The moderate reddening observed in the conjunctiva was observed in all animals after 1 hour and persisted for 24 hours in two animals. A slight ocular discharge was evident in all animals at the 1 hour reading; all effects had fully reversed by the end of the observation period (day 7). Such minimal signs of irritation observed in this study would not be expected to increase to a level that would require classification with a minor increase in the content of the *anti* isomer (i.e. from 7 to 15% as in the approved isopyrazam specification).

Comparison with the criteria

The scores for corneal opacity, iritis, conjunctival redness and conjunctival oedema were below those that would trigger classification. Furthermore, all effects had fully reversed by day seven of treatment. Therefore, RAC supports the DS's proposal for **no classification of isopyrazam for eye damage/irritation.**

RAC evaluation of respiratory sensitisation

Summary of the Dossier Submitter's proposal

The DS proposed no classification of isopyrazam for respiratory sensitisation based on lack of data.

Comments received during consultation

No comments were received during consultation.

Assessment and comparison with the classification criteria

Comparison with the criteria

RAC notes that: i) there are no data indicating evidence of respiratory tract irritation with isopyrazam; ii) the acute inhalation study showed no evidence of respiratory system impairment; and iii) rabbit dermal and eye irritation studies indicated lack of irritant potential on skin and mucosal membranes. Overall, RAC supports the DS's proposal for **no classification of isopyrazam for respiratory sensitisation.**

RAC evaluation of skin sensitisation

Summary of the Dossier Submitter's proposal

DS proposed classification of isopyrazam as Skin Sens. 1B (H317: may cause an allergic skin reaction) based on the results of a one guideline and GLP compliant murine local lymph node assay (LLNA) conducted using the 93:7 *syn*:*anti* isopyrazam. In this study, a stimulation index of 5.2 was recorded using 60% isopyrazam and an EC_3 >25% was estimated.

Comments received during consultation

One MSCA supported the DS's proposal for classification of isopyrazam as Skin Sens. 1B (H317).

Assessment and comparison with the classification criteria

Table 4 summarises the result of the murine LLNA found in the CLH report.

Table 4: Summary of the animal studies on skin sensitisation with isopyrazam.

Study	Dose level	Results	Reference
Mouse LLNA OECD 429 GLP CBA/Ca/Ola/Hsd mice 4 females/dose	Isopyrazam (batch: SMU6AP001) Purity: 96.4% (w/w) ***Syn*:*anti* ratio: 93:7** Vehicle: dimethyl sulphoxide 10, 25 and 60% (w/v)	8 lymph nodes/concentration Conc (% w/v) / Stimulation index: 10 / 2.1 25 / 1.9 60 / 5.2 The positive control gave the expected response **EC_3 value > 25%**	Anonymous (2016)

In a well conducted, guideline and GLP compliant mouse LLNA a stimulation index ≥3 was found in the 60% dose-group (stimulation index of 5.2); while isopyrazam concentrations of 10 and 25% generated stimulation indexes in both cases of around 2 (Table 4).

Comparison with the criteria

In accordance with the CLP criteria, a substance is classified as a skin sensitiser if data derived from a mouse LLNA conducted in accordance with OECD 429 produces a stimulation index of ≥3; as was the case for isopyrazam, for which a concentration of 60% (w/v) resulted in a stimulation index value of 5.2. Thus, the classification of isopyrazam as skin sensitiser is warranted. On the other hand, an EC_3 value (the concentration that results in a stimulation index value of 3) of > 2% would result in a 1B categorisation. In the study with isopyrazam, it can be concluded that the EC_3 value is greater than 25% because this concentration yielded a stimulation index of 1.9. The batch of isopyrazam tested in this investigation contained 7% of the *anti* isomer, whilst the isopyrazam specification that has been approved for use as an active substance in plant protection products contains a maximum of 15% of the *anti* isomer. The results of this skin sensitisation study indicate that an increase of the *anti* isomer to 15% would not be expected to substantially increase the skin sensitising potential of isopyrazam. Overall, RAC supports the DS's proposal for **classification of isopyrazam as Skin Sens 1B (H317: may cause an allergic reaction).**

RAC evaluation of specific target organ toxicity – repeated exposure (STOT RE)

Summary of the Dossier Submitter's proposal

DS proposed no classification of isopyrazam. The data package contained several repeated dose toxicity studies (28-day, 90-day, 1-year, 1.5-year and 2-year studies) with preparations of

isopyrazam with different *syn*:*anti* ratios. Three different species (rats, mice and dogs) were used. In his assessment the DS only noted adaptive responses of liver (increases of relative liver weight, hypertrophy and minor alterations in biochemical and haematological parameters).

Comments received during consultation

One MSCA supported the DS's proposal for no classification of isopyrazam for STOT SE.

Assessment and comparison with the classification criteria

Both EFSA and the USA EPA in their opinions, report hepatocellular hyperthophy as a substance related effect in repeated dose toxicity studies of isopyrazam.

According to the US EPA: *"Subchronic and chronic oral toxicity studies in the rat, mouse, rabbit and dog demonstrate that the primary target organ for isopyrazam is the liver (increased organ weight and centrilobular hepatocyte hypertrophy). Liver toxicity is usually accompanied by reductions in bodyweight and food consumption"*

EFSA also identified liver as the target organ of isopyrazam upon short-term and long-term exposure in all species tested: the rat, mouse, dog and rabbit.

Based on data in the CLH report, Tables 5 (See Annex 1), 6 and 7 summarise the main findings in the repeated dose toxicity studies in rats, mice and dogs; respectively.

The database contains three different 28-day oral repeated dose toxicity studies in rats with different isopyrazam preparations (*syn*:*anti* ratios 93:7, 89:11 and 50:50 and pure *anti* and pure *syn* isomers) (Table 5 – see Annex 1 at the end of the Opinion). The three studies yielded a consistent array of effects consisting in increases of relative liver weight and slight alterations in certain haematological and biochemical parameters. These alterations were in general inconsistent among different studies except for the increases in cholesterol concentrations and decreases in triglyceride concentrations. There were no treatment-related macroscopic necropsy findings. However, treatment-related microscopic changes (hepatocellular hypertrophy) was observed for all three test material ratios. Thus, these changes are likely to be an indication of an adaptive response rather than of adversity.

The two different 90-days oral repeated toxicity studies in rats with different isopyrazam preparations (*syn*:*anti* ratios 93:7 and 70:30) yielded effects quite similar to those reported for the 28-days studies (Table 5). These are, moderate increases in relative liver weight, moderate changes in blood parameters and hepatocellular hypertrophy. These are considered by RAC as adaptive changes and therefore not warranting classification. The main difference from the 28-day studies was the moderate increases in thyroid weight (Table 5). However, RAC notes that this effect was not associated to histopathological alterations and was not consistently found in all studies in rats. RAC considers this alteration as incidental and not able to warrant classification.

The neurotoxicity study in rats (Table 5) reported a number of small, isolated and inconsistent effects hence not related to treatment with isopyrazam. Therefore, these effects cannot be considered by RAC for supporting a classification.

The 2-generation toxicity study in rats also reports increases in liver weight (up to 31% at the top dose and up to 10% at the mid and low doses) and increases of incidences in hepatocellular hypertrophy (always minimal to slight) with fine cytoplasmic vacuolation (see Table 21). RAC notes that the effects at the top dose (between 217 and 700 mg/kg bw/day depending whether premating, gestation or lactation periods, see Table 21) occur at doses which are above the guidance value for setting classification based on a 2-generation toxicity study. The increases in

liver weight and hepatocellular hypertrophy at mid and low doses were minimal and were considered by RAC adaptive and therefore not sufficient for supporting classification.

Finally, the chronic toxicity study (2-years carcinogenicity study) also showed liver as the target organ of isopyrazam due to increases in liver weight and hypertrophy and vacuolation (Table 5). Again, RAC notes that these changes can be interpreted as adaptive rather than adverse and therefore will not be considered for setting classification as STOT RE. Bile duct also presented microscopic alterations (fibrosis and hyperplasia, Table 5). However, the incidences, with respect to the control, were significantly different only for males and dose-response for these alterations was unclear (especially for hyperplasia). Moreover, some cases were also reported in controls and this effect was not detected in other species or in other studies of different duration in rats. Examination of all these data allows RAC to conclude that these effects on bile ducts are likely incidental and more associated to aging than to isopyrazam exposure. Therefore, bile duct alterations will not be considered for classification.

Overall, RAC notes that no relevant effects for STOT RE classification purposes were detected in the 28-day, 90-day and 2-year repeated dose toxicity studies in rats.

Table 6: Summary for repeated dose toxicity study in mice with isopyrazam.
In all cases the difference with controls were statistically significant for at least $p<0.05$.

Method	Results	Reference
90-day oral (dietary) OECD 408 GLP C57BL/10JfCD-1 mice 10/sex/group Isopyrazam (batch: SMU6AP001) Purity: 96.4% **93:7 *syn*:*anti*** 0, 500, 2500 and 7000 ppm Males: 0, 76, 391 and 1383 mg/kg bw/day Females: 0, 87, 449 and 1760 mg/kg bw/day	There were no treatment-related deaths or clinical signs of toxicity <u>1383/1760 mg/kg bw/day</u> Moderate hepatocellular hypertrophy in males (10/10) and females (10/10) | | males | females | | ↓ Final body weight | 5% | 8% | | ↓ Albumin/Globulin ratio | - | 14% | | ↑ Alanine aminotransferase | - | 90% | | ↑ Triglycerides | - | 210% | | ↑ Relative liver weight | 47% | 59% | <u>391/449 mg/kg bw/day</u> Minimal/slight hepatocellular hypertrophy in males (10/10) and in females (10/10) | | males | females | | ↓ Final body weight | 5% | 6% | | ↑ Relative liver weight | 25% | 32% | <u>76/87 mg/kg bw/day</u> Minimal/slight hepatocellular hypertrophy in males (10/10) | | males | females | | ↑ Relative liver weight | 8% | 8% |	Anonymous (2008a)
Chronic toxicity and carcinogenicity in the mouse OECD 451	There were no treatment-related deaths. <u>433/554 mg/kg bw/day</u> | | males | females | | ↓ Body weight | 23% | 11% | | ↑ Liver weights | 41% | 38% |	Anonymous (2008b)

GLP C57BL/10JfCD-1 mice 50/sex/group 80-weeks Isopyrazam (batch: SMU6AP001) Purity: 96.4% (w/w) **93:7 *syn*:*anti*** 0, 70, 500 and 3500 ppm Males: 0, 8, 56 and 443 mg/kg bw/day Females: 0, 10, 75 and 554 mg/kg bw/day	Eye discharge	24/26	
	Hepatocellular hypertrophy	42/50	47/50
	<u>56/75 mg/kg bw/day</u>		
		males	females
	↑ Liver weights	14%	7%
	Eosinophilic droplets in gall bladder epithelium	25/50	38/50
	Inflammation in the nasolacrimal ducts	38/50	-
	Hepatocellular hypertrophy	5/50	13/50
	<u>8/10 mg/kg bw/day</u> No adverse effects		

As in the case of rats, the 90-days and 80-week repeated dose toxicity studies in mice reported adaptive effects in liver detected in the form of increases in relative liver weight and, depending of the dose, minimal/slight/moderate hepatocellular hypertrophy (Table 6). RAC does not consider these effects relevant for classification since they can be seen as an adaptive response rather than an adverse effect.

The carcinogenicity study in mice also showed some effects of eosinophilic droplets in gall bladder epithelium of uncertain toxicological significance since they do not observe dose-response and were not noted in rats and dogs. Therefore, this effect was not considered by RAC for setting STOT RE classification.

Table 7: Summary for repeated dose toxicity studies in dogs with isopyrazam.
In all cases the difference in comparison with controls were statistically significant for at least $p<0.05$.

Method	**Results**		**Reference**
90-day oral (capsule) OECD 409 GLP Beagle dogs 4/sex/group Isopyrazam (batch: SMU6AP001) Purity: 96.4%	There were no deaths <u>300 mg/kg bw/day</u> Transient head wobble, reduced stability and reduced/increased activity in 3 males during days 2/3		Anonymous (2007)
		males	females
	↓ Final body weight	10%	12%
	↑ Platelet count (week 13)	30%	-
	↑ Alkaline phosphatase (week 13)	216%	240%
	↓ Sodium (week 13)		3%
	↓ Cholesterol (week 8)		25%
	↓ urine specific gravity		2%
	↑ Relative liver weight	25%	
	Enlarged liver	2	2

***Syn*:*anti* ratio: 93:7** 0, 30, 100 and 300 mg/kg bw/day	<u>100 mg/kg bw/day</u> Transient head wobble, reduced stability and reduced/increased activity in 1 male during days 2/3 males: ↓ Albumin (week 13) -; ↑ Relative liver weight 13% females: ↓ Albumin (week 13) 5% <u>30 mg/kg bw/day</u> No adverse effects	
90-day oral (capsule) OECD 409 GLP Beagle dogs 4/sex/group Isopyrazam (batch: SMU7DP017) Purity: 96.4% **70:30 *syn*:*anti*** 0, 10, 30 and 250 mg/kg bw/day	There were no deaths <u>250 mg/kg bw/day</u> ↑ salivation in all dogs, abnormal behaviour in 1 male from week 4 (sedation, shaking head, buckling of hind limbs, uncoordinated movements and ptosis) 4% body-weight loss in 1 male in week 1 10% body-weight loss in 1 female in wek 1 <u>30 mg/kg bw/day</u> No adverse effects <u>10 mg/kg bw/day</u> No adverse effects	Anonymous (2008a)
12-month oral (capsule) OECD 452 GLP Beagle dogs 4/sex/group Isopyrazam (batch: SMU6AP001) Purity: 96.4% **93:7 *syn*:*anti*** 0, 25, 100 and 250 mg/kg bw/day	There were no deaths or treatment-related clinical signs of toxicity <u>250 mg/kg bw/day</u> males: ↓ Body weight gain in 3 males 8%, 3%, 7%; ↓ Body weight gain in 2 females (week 1) -; ↓ Body weight gain (week 52) 57%; ↑ Alkaline phosphatase (week 52) 415%; ↑ Alanine aminotransferase (week 52) 294%; ↑ Glutamate dehydrogenase (week 52) 830%; ↓ Bilirubin (week 52) 48%; ↓ Albumin (week 52) 13%; ↑ Liver weight 52% females: ↓ Body weight gain in 3 males -; ↓ Body weight gain in 2 females (week 1) 6%, 4%; ↓ Body weight gain (week 52); ↑ Alkaline phosphatase (week 52) 272%; ↑ Alanine aminotransferase (week 52) -; ↑ Glutamate dehydrogenase (week 52) -; ↓ Bilirubin (week 52) -; ↓ Albumin (week 52) 10%; ↑ Liver weight -	Anonymous (2008b)

100 mg/kg bw/day

	males	females
↑ Alkaline phosphatase (week 52)	274%	243%
↓ Bilirubin (week 52)	48%	-
↑ Liver weight	42%	-

25 mg/kg bw/day

	males	females
↑ Alkaline phosphatase (week 52)	223%	-

The 90-day oral repeated dose toxicity study in dogs with the isopyrazam preparation 70:30 *syn*:*anti* showed only transient clinical effects without apparent liver impairment (Table 7). However, the 90-days and 12-month repeated dose toxicity studies in dogs with the isopyrazam preparation with *syn*:*anti* ratio 93:7 showed an array of effects compatible with those reported in the repeated dose toxicity studies in rats and mice where liver was detected as the main target organ of isopyrazam. The effects in dogs were increases in relative liver weight together with changes in blood parameters (Table 7). The marked changes in these blood parameters were associated to transaminases and are considered secondary to liver changes. Unlike the studies in rats and mice, liver hypertrophy was not reported in dogs and therefore RAC will not consider hepatic changes for warranting STOT RE classification.

In addition to the effects reported above in rats, mice and dogs, RAC notes that a teratogenicity study in rabbits (Anonymous 2008b) also reports minimal to mild centrilobular hepatocellular hypertrophy/vacuolation in liver at 150 and 500 mg/kg bw/day together with increases in liver weight of 13 and 36%. The top dose of this developmental study (500 mg/kg bw/day) was outside the limit concentration for setting classification based on a developmental toxicity study in rabbits where the exposure is for 22 days. The effects at 150 mg/kg bw/day (minimal hypertrophy and 13% increase in liver weight) are considered by RAC again as indicative of an adaptive liver response and not susceptible to warrant a classification as STOT RE.

Comparison with the criteria

Classification for STOT-RE is assigned on the basis of 'significant' or 'severe' toxicity. Significant is defined as changes which clearly indicate functional disturbance or morphological changes which are toxicologically relevant, whilst severe effects are generally more profound and are of a considerably adverse nature and likely to have a significant impact on health. None of the effects observed for isopyrazam fulfilled any of these criteria. On the contrary, several effects were noted which were specifically considered in the Guidance on the Application of the CLP Criteria as effects not supporting classification as STOT RE. These effects were:

1. *Clinical observations or small changes in body-weight gain.* Clinical observations were noted in dogs comprising transient head wobble, reduced stability and increased or decreased activity. Small reductions in body-weight gain were noted in rats (<10%) but not in dogs or mice.
2. *Changes in organ weights with no evidence of organ dysfunction.* Liver weight increases were the main feature of isopyrazam exposure; in rats, at the relevant doses for classification into category 2, relative liver weights were increased by up to 30% and 25% in males and females respectively. In mice, the magnitude of the liver weight increases was 47% and 59% in males and females respectively after 90-days' exposure, whilst liver weights in male dogs were increased by up to 52% after 12-months' exposure (the liver

weights of female dogs were not affected). There were no accompanying signs of organ dysfunction.

3. *Adaptive responses that are not considered toxicologically relevant.* The liver weight increases observed in rats and mice after administration of isopyrazam were accompanied by findings indicative of an adaptive response; hepatocellular hypertrophy was noted in both sexes and analysis of enzyme activity showed an increase in total P450 activity (particularly PROD).

In conclusion, in rats, mice and dogs the only effects observed after the administration of isopyrazam (at the relevant doses for classification) were clinical observations, small changes in body-weight gain, small changes in biochemistry parameters and changes in organ weights (increased liver weights) which were accompanied by histopathological findings (centrilobular hepatocyte hypertrophy) indicative of an adaptive response. Overall, RAC supports the DS's proposal for **no classification of isopyrazam for STOT RE.**

RAC evaluation of germ cell mutagenicity

Summary of the Dossier Submitter's proposal

The gene mutation potential of both the 93:7 and the 70:30 *syn*:*anti* ratios of isopyrazam has been investigated *in vitro* in bacteria (Ames tests) and in mammalian cells (mouse lymphoma). The clastogenic potential of both specifications was investigated *in vitro* in human lymphocytes. The potential of the 93:7 *syn*:*anti* specification of isopyrazam to induce chromosomal damage in rats has been investigated *in vivo* in a bone marrow micronucleus test and in an unscheduled DNA synthesis assay. All *in vivo* and *in vitro* tests yielded negative results hence the DS proposed no classification of isopyrazam for germ cell mutagenicity.

Comments received during consultation

One MSCA supported the DS's proposal for no classification of isopyrazam for germ cell mutagenicity.

Assessment and comparison with the classification criteria

Tables 8 and 9 summarise the *in vitro* and *in vivo* mutagenicity studies with isopyrazam; respectively.

Table 8: Summary of mutagenicity/genotoxicity in vitro studies with isopyrazam.

Method	Tested concentrations	Results	Reference
Bacterial reverse mutation assay *S. typhimurium* strains TA1535, TA1537, TA98 and TA100	Isopyrazam (batch: SMU6AP001) Purity: 96.4% (w/w) **93:7 *syn*:*anti*** 5-5000 µg/plate	Precipitation at 500-5000 µg/plate Cytotoxicity at 2500 and 5000 µg/plate Appropriate positive and solvent controls gave the expected results Tested in triplicate **Results:** **+S9: Negative** **- S9: Negative**	Callander (2006)

E.coli strains WP2 (pKM101) and WP2 uvrA (pKM101) OECD 471 GLP			
Bacterial reverse mutation assay *S. typhimurium* strains TA1535, TA1537, TA98 and TA100 *E. coli* strains WP2 (pKM101) and WP2 uvrA (pKM101) OECD 471 GLP	Isopyrazam (batch: SMU7DP017) Purity: 90.8% (w/w) **70:30 *syn*:*anti*** 5-5000 µg/plate	Precipitation at 333-5000 µg/plate No cytotoxicity up to 5000 µg/plate Appropriate positive and solvent controls gave the expected results Tested in triplicate **Results:** **+S9: Negative** **- S9: Negative**	Sokolowski (2008a)
In vitro mammalian cell gene mutation test Mouse lymphoma cells, L5178Y TK+/- locus OECD 476 GLP	Isopyrazam (batch: SMU6AP001) Purity: 96.4% (w/w) **93:7 *syn*:*anti*** Max 40 µg/ml (+S9) Max 25 µg/ml (-S9)	Cytotoxicity: relative total growth at highest concentration was 13% (+S9) and 19% (-S9) Positive controls induced the appropriate increases in mutant frequencies Each concentration tested in duplicate (2 independent experiments) **Results:** **+S9: Negative** **- S9: Negative**	Clay (2006)
In vitro mammalian cell gene mutation test Mouse lymphoma cells, L5178Y TK+/- locus OECD 476	Isopyrazam (batch: SMU7DP017) Purity: 90.8% (w/w) **70:30 *syn*:*anti*** Max: 66 µg/ml (+S9) Max: 44 µg/ml (-S9)	Precipitation from 175 µg/l Cytotoxicity: minimum survival levels (compared with controls) at highest concentration: 8% (+/-S9) Positive controls induced the appropriate increases in mutant frequencies **Results:** **+S9: Negative** **- S9: Negative**	Wollny (2008a)

In vitro chromosome aberration test Human lymphocytes: 2 donors (1 male and 1 female, pooled) OECD 473 GLP	Isopyrazam (batch: SMU6AP001) Purity: 96.4% (w/w) **93:7 *syn*:*anti*** <u>Assay 1:</u> 20-40 µg/ml (-S9) and 20-50 µg/ml (+S9) 3 h exposure (+/-S9) <u>Assay 2:</u> 10-20 µg/ml (-S9) and 20-50 µg/ml (+S9) 3 h exposure (+/-S9)	Cytotoxicity: >50% reduction in mitotic activity at the highest concentration (concentration dependent) Small increases in the % of aberrant cells in experiment 1 (-S9 at 20 and 30 µg/ml) were within historical control ranges and there were no increases at the highest concentration of 40 µg/ml Positive and negative controls gave the expected results **Results assay 1:** **+S9: Negative** **-S9: Negative** **Results assay 2:** **+S9: Negative** **-S9: Negative**	Fox (2006a)
In vitro chromosome aberration test Human lymphocytes: 2 donors (1 male and 1 female, pooled) OECD 473 GLP	Isopyrazam (batch: SMU7DP017) Purity: 90.8% (w/w) **70:30 *syn*:*anti*** <u>Assay 1:</u> 16.9-51.7 µg/ml (-S9) and 29.6-90.5 µg/ml (+S9) 4h exposure (+/- S9) <u>Assay 2:</u> 3-16 µg/ml (-S9) and 25-75 µg/ml (+S9) 4h exposure (+S9) and 22h exposure (-S9)	Cytotoxicity: >50% concentration-dependent reduction in mitotic activity at the highest concentrations Positive and negative controls gave the expected results **Results assay 1:** **+S9: Negative** **- S9: Negative** **Results assay 2:** **+S9: Negative** **-S9: Negative**	Bohnenberger (2008a)

The application of isopyrazam to *Salmonella typhimurium* and *Escherichia coli* strains, up to and including the limit concentration of 5000 µg/plate, did not produce an increase in the number of reversions either with or without metabolic activation. The potential of isopyrazam to induce gene mutations was further investigated *in vitro* at the TK locus of L5178 mouse lymphoma cells and was found to be negative both with and without S9 mix, when tested up to appropriate cytotoxic and precipitative concentrations. Furthermore, isopyrazam showed no evidence of clastogenic potential *in vitro* in human peripheral blood lymphocytes both in the presence and absence of S9 mix, even when tested up to cytotoxic concentrations.

Table 9: Summary of mutagenicity/genotoxicity in vivo studies with isopyrazam.

Method	Tested concentrations	Results	Reference
In vivo rat bone marrow micronucleus test OECD 474	Isopyrazam (batch: SMU6AP001) Purity: 96.4% (w/w) **93:7 *syn*:*anti***	2000 erythrocytes examined No clinical signs of toxicity noted **Negative**	Anonymous (2006b)

GLP HsdRCCHan:WIST rats 5 males	2000 mg/kg bw, oral (limit dose) Bone marrow sampled at 24 and 48 hours		
In vivo rat Liver unscheduled DNA Synthesis Assay OECD 486 GLP HsdRCCHan:WIST rats 3 males	Isopyrazam (batch: SMU6AP001) Purity: 96.4% (w/w) **93:7 *syn*:*anti*** 2000 mg/kg bw, oral (limit dose) Hepatocytes sampled at 2 and 16 hours	Hepatocytes sampled at 2 and 16 hours No clinical signs of toxicity noted No histopathological changes to the liver Positive control induced marked increase in unscheduled DNA synthesis **Negative**	Anonymous (2006c)

Isopyrazam (93:7 *syn*:*anti*) was investigated for its ability to induce micronucleated immature erythrocytes in the bone marrow of male HsdRCCHan:WIST rats. There were no increases in the incidences of micronucleated immature erythrocytes (when compared with vehicle control values) at either sampling time; a biologically meaningful increase in micronuclei was induced by the positive control, thus demonstrating the sensitivity of the assay. Exposure of the bone marrow to the test substance was demonstrated by a reduction in the mean percentage of immature erythrocytes in the treated groups compared with the vehicle control groups. Under the conditions of this study, isopyrazam was not clastogenic *in vitro* in rats.

Isopyrazam (93:7 *syn*:*anti*) did not induce unscheduled DNA synthesis in the livers of rats in an *in vivo* unscheduled DNA synthesis study. A single oral limit dose of 2000 mg/kg bw did not result in any changes to the mean net nuclear grain counts or the % of DNA repair in hepatocytes sampled at 2 and 16 hours post-dose. The positive control (N-nitrosodimethylamine) induced marked increases in unscheduled DNA synthesis demonstrating the sensitivity of the assay.

Comparison with the criteria

There is no evidence from the available data set that isopyrazam is a somatic cell mutagen. Moreover, there is no reason to believe that isopyrazam would have the potential to induce mutations in germ cells. Therefore, RAC supports the DS's proposal for **no classification of isopyrazam for germ cell mutagenicity.**

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

The DS presented two carcinogenicity studies in the CLH report, one in mice and one in rats. The study in mice showed no neoplastic findings; while the study in rats showed neoplastic findings in thyroid, liver, uterus, mammary gland and thyroid gland. Only uterine and hepatocellular tumours were considered as treatment-related. However, for both types of tumours, the CLH report presents specific studies conducted for establishing modes of action not relevant for humans. In conclusion, the DS proposed no classification of isopyrazam for carcinogenicity.

Comments received during consultation

One company/manufacturer agreed with the DS's proposal for no classification of isopyrazam for carcinogenicity and submitted attachments with additional information. This same company/manufacturer released a second similar comment providing a summary of the submitted documents in the text. DS thanked and replied that this additional information should be considered by RAC. RAC notes that the position paper entitled *"Isopyrazam-Human Relevance Framework Assessment of Liver Tumour Induction in Female Rats"* was prepared with the aim to assess a hypothesised mode of action for isopyrazam-induced liver tumours using the framework developed by the International Programme on Chemical Safety (IPCS) and the International Life Science Institute (ILSI). RAC has taken this paper into consideration in its assessment but notes that is quite similar to Annex 2 of the CLH report.

One MSCA raised concerns about the relevance of liver tumours for humans considering that the key events relationships of the constitutive androstane receptor (CAR) mediated mode of action have not been established by a weight of evidence analysis. Moreover, this MSCA also raised the question that alternative modes of actions such as sustained aryl hydrocarbon receptor (AhR) activation leading to rodent liver tumours; inhibition of inducible nitric oxide synthase (iNOS), hepatotoxicity, and regenerative proliferation leading to liver tumours; or mutagenic mode-of-action leading to hepatocellular carcinoma have not been ruled out. Overall, this MSCA considered that classification as Carc. 2 is more appropriate than the no classification proposed by the DS.
A second MSCA also proposed classification of isopyrazam as Carc. 2 in line with the argumentation for classification of sedaxane, as sedaxane and isopyrazam are structural analogues and both induce liver and uterine tumours. Moreover, this MSCA also demanded a careful analysis of isopyrazam carcinogenic effects in the light of the succinate dehydrogenase inhibition. The DS replied that a robust assessment of the data on isopyrazam had not been presented at the time of assessment of sedaxane by RAC and now the full data on isopyrazam should be given due consideration. The classification of isopyrazam cannot be automatically extrapolated from the sedaxane classification because the database is not identical and because isopyrazam contains some mechanistic studies not previously considered for the case of sedaxane; for example, the mechanistic 18-month study in rats. RAC supports the DS's view that the data should be given full consideration, however, notes that isopyrazam mechanistic information including the 18-months study and evaluation of hypothalamic tyrosine hydroxylase in samples from 13-week dietary and 2-year carcinogenicity studies, was indeed submitted also for sedaxane assessment. The same Mode of action for uterine carcinoma was claimed for both substances. The 18-month investigative isopyrazam study was submitted as new data, with a detailed weight of evidence document describing the Mode of action and human non-relevance of uterine tumours. At that time RAC carefully looked at all information provided, including the isopyrazam data, to understand the proposed Mode of action hypothesis.

The DS also replied that there is no convincing evidence that succinate dehydrogenase inhibitors induce liver or uterine tumours since a genetic defect of succinate dehydrogenase gene in humans lead to encephalopathies and cardiomyopathies in addition to the fact that isopyrazam is extensively metabolised, and therefore it is unlikely that it is able to induce a sustained inhibition over time.

A third MSCA also raised concerns about the capability of succinate dehydrogenase inhibitors to induce uncontrolled proliferation of cells causing cancer. This same MSCA also raised concerns about the uncertainties that remains regarding the mode of action of isopyrazam for induction of liver carcinomas. The concerns being based on the lack of data from *in vitro* CAR/PXR assay, gene expression, CAR-knock out animals and the fact that hepatocytes from a single donor were used.

Finally, the MSCA highlighted uncertainties also regarding the mode of action of isopyrazam for induction of uterine tumours. These uncertainties were addressed towards key events 3, 5 and 6. In conclusion, the MSCA considered that the classification of isopyrazam as Carc. 2 is warranted. The DS replied in similar terms to those stated above for the second MSCA.

A fourth MSCA expressed concerns on all the justifications provided by the DS questioning whether the induction of CYP could be caused as an adaptative response of the liver, the target organ of isopyrazam, the mode of action behind these carcinogenic effects And whether all relevant uncertainties have been clarified?

Assessment and comparison with the classification criteria

The US EPA classifies isopyrazam as "*Likely to be Carcinogenic to Humans*" based on increased incidence of uterine endometrial adenocarcinomas and liver hepatocellular adenomas in female rats and increased incidence of thyroid follicular cell adenomas and/or carcinomas in male rats.

According to the EFSA opinion 2012 the long-term exposure to isopyrazam produces liver hepatocellular adenomas and uterine endometrial adenocarcinomas in rats and therefore proposed the classification as a carcinogen category 3, R40 "*limited evidence of a carcinogenic effect*".

Carcinogenicity study in mice (Anonymous 2008b)

Isopyrazam (93:7 *syn*:*anti*) was administered to male and female mice for 80-weeks at doses of 0, 70, 500 and 3500 ppm, which equals to mean intakes of 0, 8, 56 and 433 mg/kg bw/day in males and 0, 10, 75 and 554 mg/kg bw/day in females. There was no effect on the survival rate of the mice and the only treatment-related sign of toxicity was a discharge from the eyes of the high dose males. Table 6 summarises the non-neoplastic findings of this study. Exposure to isopyrazam had no effect on the incidence, appearance or onset of tumours in male or female mice.

Two-year dietary toxicity and carcinogenicity study in rats and histological extension (Anonymous 2008a and Anonymous 2009)

In a two-year combined chronic and carcinogenicity study in rats, isopyrazam 93:7 *syn*:*anti* was administered for either 52 (12/sex/group) or 104 (52/sex/group) weeks at dietary concentrations of 0, 100, 500 and 3000 ppm. These doses equal mean intakes of 0, 5.5, 28 and 173 mg/kg bw/day in males and 0, 7, 35 and 233 mg/kg bw/day in females. Table 5 summarises the non-neoplastic findings of this study.

Neoplastic findings

There were no treatment-related neoplastic findings at the interim kill (52 weeks). In the main study (104 weeks), there was a dose-dependent increase in the incidence of uterine adenocarcinomas (low-to high dose groups) and an increase in liver hepatocellular adenomas in females at 3000 ppm (233 mg/kg bw/day). In males, there was an increase in the incidence of thyroid follicular cell adenoma at the high dose.

Thyroid follicular cell adenoma

The incidences of thyroid cell tumours are shown in the Table 10. The incidence of thyroid follicular cell adenomas in males at 3000 ppm was 12%; which is statistically significantly higher than that of the concurrent controls. However, there was no evidence of a dose response (total incidence was 1/52, 4/52, 2/52 and 6/52 at 0, 100, 500 and 3000 ppm respectively). Furthermore, no increase in carcinomas was noted. The historical control data (HCD) available provided a range of 1.6% to 9.6% based on just three previous carcinogenicity studies (conducted between 2007-2009).

Table 10: Thyroid follicular cell tumours in the 2-year carcinogenicity study in rats.

Parameter	Dietary isopyrazam (ppm) 0	100	500	3000	HCD
Number of organs examined		52	52	52	52	
Males					
Adenoma		1	4	2	6 (12%)*	Performing facility: 1.6-9.6% (3 studies 2007-2009)
Carcinoma		0	0	5	0	
Females					
Adenoma		5	1	3	3	
Carcinoma		0	1	0	0	

*Statistically significant increase, p not available.

RAC does not consider thyroid follicular cell adenomas in males relevant for classification purposes based mainly on the lack of dose-response and supported by the fact that the increase of incidence is only marginally outside the values for the laboratory's historical control data.

Hepatocellular adenoma

The incidences of liver tumours are shown in the Table 11. The incidence of hepatocellular adenomas was increased at 3000 ppm in females (11/52 (21%) compared with 0/52 in the control group) and a few adenomas were reported for the high dose males. Statistical assessment has not been provided in the CLH report, however, the incidence in females is clearly above the HCD of the performing facility. There are also single incidences of hepatocellular carcinoma in the high dose males and females, while the HCD incidence for the carcinoma is zero. A non-genotoxic mode of action for isopyrazam-induced liver hepatocellular adenomas has been proposed (see sections below).

Table 11: Hepatocellular tumours in the 2-year carcinogenicity study in rats. Statistical assessment was not provided.

Parameter	Dietary isopyrazam (ppm) 0	100	500	3000	HCD
Number of organs examined	52	52	52	52	
Males					
Adenoma	1	0	0	3	
Carcinoma	0	0	0	1	
Females					
Adenoma	0	1	1	11 (21%)	Performing facility: 0-1.9% (3 studies 2007-2009)
Carcinoma	0	0	0	1	0% (3 studies 2007-2009)

Uterine endometrial adenocarcinoma

The incidence of uterine tumours is shown in Table 12. The incidences of uterine endometrial carcinoma in females were zero for all groups at the interim sacrifice (12 animals/group), while at terminal sacrifice of the 52 animals a dose-dependent increase with 1/52, 2/52, 3/52, 15/52 for control, low, mid and high dose group, respectively, is reported, the high dose of 3000 ppm shows an incidence of 29%); the incidence in the control animals was just 1/52 at terminal kill. Statistical assessment has not been provided in the CLH report. HCD of the performing laboratory from the appropriate time period provides a substantially lower upper incidence for this carcinoma in Wistar rats (range 1.9-7.8%, no mean available) and there is no indication that the concurrent control group is not reliable and thus considered the most relevant control for the assessment for the findings. A non-genotoxic mode of action has been proposed for this tumour (see sections below).

Table 12: Uterine tumours in the 2-year carcinogenicity study in rats. Statistical assessment was not provided.

Parameter	Dietary isopyrazam (ppm) 0	100	500	3000	HCD
Number of organs examined	52	52	52	52	
Adenoma	1	0	1	0	
Carcinoma	1	2	3	15 (29%)	Performing facility: 1.9-7.8% (3 studies 2007-2009)

Other neoplastic incidences

The Table 13 provides a summary of neoplastic findings in mammary and pituitary glands. In both organs, the incidences of both adenomas and carcinomas was not statistically different from the incidences of controls. No tumour induction and thus no concern is identified for these two glandular tissues. RAC notes that tumour incidences were reduced at the mid and high doses. The observation that mammary and pituitary gland tumour incidences were concomitantly reduced with the increased uterine tumour incidences, is a similar finding as for the structural analogue sedaxane, and thus it may be treatment-related. The reduction of mammary and pituitary gland tumour incidence is considered by the applicant a relevant and treatment related tumour outcome in the Mode of action hypothesis for uterine adenocarcinomas induced by isopyrazam.

Table 13: Mammary and pituitary tumours in the 2-year carcinogenicity study in rats.

Parameter	Dietary isopyrazam (ppm) 0	100	500	3000	HCD
Number of organs examined	52	52	52	52	
Mammary tumours - Females					
Adenoma	14	16	9	4	Not available
Carcinoma	2	7	5	0	Not available
Pituitary tumours - Females					
Adenoma	32	33	28	24	Not available
Carcinoma	0	1	0	0	Not available

Conclusion:

Overall, RAC concludes that the increases in the incidence of hepatocellular adenoma and carcinomas in males and females and the uterine carcinoma with a dose-response relationship are treatment related. The genotoxicity profile for this substance is negative (see section about germ cell mutagenicity), suggesting a non-genotoxic mode of action.

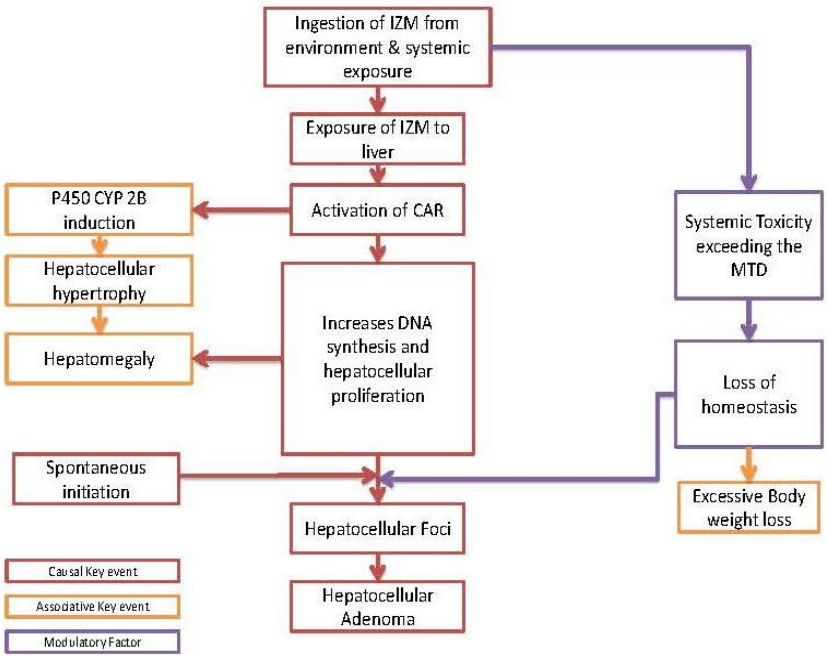
PROPOSED MODE OF ACTION FOR LIVER HEPATOCELLULAR ADENOMAS

There are various possible mechanistic explanations for this weak carcinogenic response in high dose female rats, including genotoxicity, peroxisome proliferator activated receptor alpha (PPARα) receptor activation, AhR receptor activation, CAR/PXR receptor activation, oestrogenic stimulation, statins, cytotoxicity, immunosuppression, porphyria and increased apoptosis.

Annex 2 to the CLH report contains a document provided by the applicant describing the mode of action and the human health relevance assessment of the increased incidence of liver tumours in female Han Wistar rats dosed with isopyrazam. This document outlines a phenobarbital-like mode of action that involves the activation of the CAR as the most likely cause. This mechanism is generally accepted to be implausible in humans on a qualitative basis (Elcombe et. al, 2014).

The proposed Mode of action for the development of liver tumours in female rats is summarised below:

IZM = isopyrazam



After exposure of the liver to sufficiently high free concentrations of isopyrazam from the systemic circulation, activation of the CAR, NR1I results in an increased expression of its target genes' messenger RNA (mRNA), including those that regulate xenobiotic metabolism, DNA proliferation and the cell cycle. Consequently, it is proposed that isopyrazam activated CAR results in increased DNA synthesis, increased proliferation and ultimately hepatocellular adenoma.

Mechanistic studies: CAR activation

The CAR activation step of the mode of action also induces a variety of xenobiotic metabolism genes (e.g. P450 CYP 2B) which results in smooth endoplasmic reticulum proliferation, leading to hepatocyte hypertrophy and hepatomegaly (when combined with mitogenesis). These events associated with CAR activation serve as measurable markers for this key event in the proposed mode of action. Hence, CAR activation can be demonstrated by the measurement of these associated markers. Increased P450 Cyp2b activity (indirectly measured by activity of pentoxyresorufin O-dealkylase (PROD) and benzyloxyresorufin O-dealkylation (BROD)), hepatocellular hypertrophy and increased liver weight along with increased DNA synthesis and cell proliferation all provide supportive evidence for the proposed mode of action.

Table 14 summarises *in vitro* experiments with human and rat hepatocytes. RAC notes that isopyrazam and phenobarbital caused increases in the expression of both PROD and BROD in both rat hepatocytes and human hepatocytes (Table 14). However, isopyrazam and phenobarbital triggered cell proliferation in rat hepatocytes but not in human hepatocytes. Data contained in Table 14 suggests that the response of human hepatocytes to isopyrazam is different to the response of rat hepatocytes to this substance because, despite the activation of CAR receptor, human hepatocytes do not undergo cell proliferation. RAC notes that, despite the fact that no proliferation was noted in human hepatocytes in three different donors at three different isopyrazam concentrations (1, 10 and 30 µM), the hepatocytes coming from all these three human donors are able to proliferate when they are exposed to the positive control. RAC also notes that use of *in vitro* hepatocytes from only female rats leads to some uncertainties on the Mode of action.

Table 14: *In vitro* study for assessing enzyme induction, cytotoxicity and cell proliferation in Wistar rat and human hepatocytes

<table>
<tr><th>Test substance, test system, Reference</th><th>Conc/dose, replicates, duration of exposure</th><th>Type of data</th><th>Relevant information about the study (as applicable)
(values are compared to controls)</th></tr>
<tr><td rowspan="3">Isopyrazam (batch: SMU6AP001)

Purity: 96.4% (w/w)

93:7 syn:anti
Positive controls: Phenobarbital (PB) and epidermal growth factor (EGF)

Vehicle control: DMSO

Cultured hepatocytes from female Han Wistar Rats (same strain as the carc. study)

Cryopreserved, cultured human hepatocytes; viability > 65%

Non GLP compliance (abides to GLP principles)

Ellcombe (2011) and Ellcombe (2011a)</td><td rowspan="3">Isopyrazam: 1-100 µM

PB: 10-1000 µM

96-hours exposure

<u>CYP2B activity:</u>
3 replicates

<u>Proliferation:</u>
5 replicates, 1500 hepatocytes/conc

<u>ATP depletion:</u>
6 replicates</td><td>Cytotoxicity assay</td><td>Both rats and humans: Cytotoxicity of isopyrazam demonstrated by ATP depletion from 65 µM (effects observed at non-cytotoxic concentrations)</td></tr>
<tr><td>Enzyme Induction

CYP2B activity measured by PROD

CYP2B/CYP3A activity measured by BROD</td><td>Isopyrazam
<table><tr><th></th><th>Isopyrazam [µM]</th><th>HUMAN</th><th>RAT</th></tr><tr><td>PROD</td><td>10</td><td>-</td><td>↑ 4.1</td></tr><tr><td></td><td>30</td><td>↑ 1.7</td><td>↑ 6.6</td></tr><tr><td>BROD</td><td>1</td><td>↑ 2.9</td><td>-</td></tr><tr><td></td><td>3</td><td>↑ 3.8</td><td>↑ 1.4</td></tr><tr><td></td><td>10</td><td>↑ 4.2</td><td>↑ 2.2</td></tr><tr><td></td><td>30</td><td>↑ 4.9</td><td>-</td></tr></table>Phenobarbital
<table><tr><th></th><th>Phenobarbital [µM]</th><th>HUMAN</th><th>RAT</th></tr><tr><td>PROD</td><td>100</td><td>-</td><td>↑ 4.0</td></tr><tr><td></td><td>1000</td><td>↑ 1.8</td><td>↑ 5.8</td></tr><tr><td>BROD</td><td>100</td><td>↑ 2.0</td><td>-</td></tr><tr><td></td><td>1000</td><td>↑ 2.6</td><td>↑ 6.3</td></tr></table></td></tr>
<tr><td>Replicative DNA synthesis</td><td><table><tr><th colspan="3">Hepatocyte proliferation</th></tr><tr><th></th><th>HUMAN</th><th>RAT</th></tr><tr><td>Isopyrazam</td><td>Absent</td><td>Yes</td></tr><tr><td>PB</td><td>Absent</td><td>Yes</td></tr><tr><td>EGF</td><td>Yes</td><td>-</td></tr></table></td></tr>
</table>

Mechanistic information: In vivo enzyme induction and cell proliferation

The CLH report contains an *in vivo* study in rats devoted to determin enzyme induction and cell proliferation in rats. This study is summarised in Table 15. Two doses (one carcinogenic and the other not; 327 mg/kg bw/day and 58 mg/kg bw/day, respectively) resulted in significant increases in total microsomal CYP content comprising an increase in ethoxyresorufin O-deethylase (EROD) activity (a marker of CYP1A) and PROD activity (a marker of CYP2B). The increase in PROD activity (up to several hundred-fold of control activity levels) was much greater than the increase in EROD activity that was up to ~3-fold control activity. A large increase in EROD (CYP1A) would indicate activation of AhR rather than CAR. Activation of PPARα was discounted by the lack of a significant increase in lauric acid 12-hydroxylation (CYP4A) in the microsomes and peroxisomal palmitoyl CoA oxidase activity in the peroxisomes. No increase in testosterone 6β-hydroxylation (a marker of CYP3A activity) was noted. A two-fold increase of

liver cell proliferation was observed at the carcinogenic dose but not at the non-carcinogenic dose after three days of treatment and had returned to control levels thereafter. Overall, RAC notes that this study demonstrated that isopyrazam *in vivo* activates CAR in rat hepatocytes, suggesting the triggering of cell proliferation. RAC also notes that, although weaker, AhR activation is indicated and thus a role of AhR in cell cycle regulation and hepatocyte proliferation cannot be fully ruled out.

Table 15: *In vivo* study measuring enzyme induction and cell proliferation in rats.

<table>
<tr><th>Type of study/data, aim of study</th><th>Test substance, test system</th><th>Conc/dose, replicates, duration of exposure</th><th>Aim, results/remarks</th></tr>
<tr>
<td>Repeated dose mode-of-action study in rats

Non GLP compliance (abides to GLP principles)

Anonymous (2011)</td>
<td>Isopyrazam (batch: SMU6AP001)

Purity: 96.4% (w/w)

93:7 syn:anti
Han Wistar [CrL:WI(Han)] rats (same strain as carc. study),

10 females/dose

Peroxisomal and microsomal subcellular fractions</td>
<td>0, 58 and 327 mg/kg bw/d

3-, 7- and 14-days exposure

<u>Measurements:</u>

Peroxisome proliferation (palmitoyl co-enzyme A metabolism)

Total CYP (reduced-CO binding spectra of CYP)

CYP1A (ethoxyresorufin-O deethylase (EROD))

CYP2B (PROD)

CYP3A ([^{14}C]-testosterone 6β-hydroxylation)

CYP4A ([^{14}C]-lauric acid 12-hydroxylation)

Cell proliferation (uptake of BrdU into the nucleus of S-phase cells)</td>
<td>
General toxicity (in all cases statistically different form control for p<0.01)
<table>
<tr><th></th><th colspan="2">mg/kg bw/day</th></tr>
<tr><th></th><th>58</th><th>327</th></tr>
<tr><td>Day 3</td><td></td><td></td></tr>
<tr><td>↓ Body weight gain</td><td>-</td><td>45%</td></tr>
<tr><td>↑ Liver weights</td><td>20%</td><td>16%</td></tr>
<tr><td>↑ mitosis</td><td>-</td><td>YES</td></tr>
<tr><td>Day 7</td><td></td><td></td></tr>
<tr><td>↓ Body weight gain</td><td>-</td><td>25%</td></tr>
<tr><td>↑ Liver weights</td><td>6%</td><td>15%</td></tr>
<tr><td>Centrilobular hypertrophy</td><td>-</td><td>5 (minimal)</td></tr>
<tr><td>14-days</td><td></td><td></td></tr>
<tr><td>↑ Liver weights</td><td>10%</td><td>24%</td></tr>
<tr><td>Centrilobular hypertrophy</td><td>-</td><td>5 minimal + 5 mild</td></tr>
</table>
Enzyme induction

There were no treatment related effects on peroxisome proliferation, CYP3A or CYP4A
<table>
<tr><th></th><th colspan="2">mg/kg bw/day</th></tr>
<tr><th></th><th>58</th><th>327</th></tr>
<tr><td>Day 3</td><td></td><td></td></tr>
<tr><td>Total P450</td><td>↑ 1.8-fold</td><td>↑1.6-fold</td></tr>
<tr><td>CYP2B (PROD)</td><td>↑ 303-fold</td><td>↑ 346-fold</td></tr>
<tr><td>CYP1A (EROD)</td><td>↑ 3.3-fold</td><td>↑ 2.1-fold</td></tr>
<tr><td>Day 7</td><td></td><td></td></tr>
<tr><td>Total P450</td><td>↑ 1.5-fold</td><td>↑ 1.5-fold</td></tr>
<tr><td>CYP2B (PROD)</td><td>↑ 110-fold</td><td>↑ 162-fold</td></tr>
<tr><td>CYP1A (EROD)</td><td>↑ 2.5-fold</td><td>↑2.5-fold</td></tr>
<tr><td>14-days</td><td></td><td></td></tr>
<tr><td>Total P450</td><td>↑ 1.5-fold</td><td>↑ 1.6-fold</td></tr>
<tr><td>CYP2B (PROD)</td><td>↑ 209-fold</td><td>↑ 340-fold</td></tr>
<tr><td>CYP1A (EROD)</td><td>↑ 2.4-fold</td><td>↑ 2.7-fold</td></tr>
</table>
Proliferation

↑ liver cell proliferation after 3-days: 327 mg/kg bw/day group mean S-phase labelling index (%) was increased 2-fold after 3-days and returned to control levels at days 7 and 14

There was no evidence of cell proliferation at 58 mg/kg bw/day at any time-point
</td>
</tr>
</table>

Integration of mechanistic studies

RAC in the past discussed minimum requirements to demonstrate that a CAR Mode of action is plausible. Table 16 summarises the data made available in the case of hepatoadenomas and carcinoma induced by isopyrazam. Human hepatocytes have shown that CAR is also activated, although presumably at a lower level than in rats, but the key event of cellular proliferation that triggers downstream hypertrophy, altered foci and carcinomas does not take place (Table 15). Importantly, *in vivo* CAR knock-out studies demonstrating lack of enzyme expression, replicative DNA synthesis and liver tumour promotion have not been conducted, which leaves uncertainties in the Mode of action hypothesis.

Table 16: Isopyrazam datasets to establish a CAR-mediated mode of action for rodent liver tumors

EVENT	RATS	HUMAN
CAR activation based on *in vitro* and *in vivo* enzyme expression studies: Increased CYP2b, Cyp3a	**YES** (Table 10)	**YES** (Table 10)
Hepatocellular proliferation	**YES** (Table 14, Table 15)	**NO** (Table 14) -
Hepatocellular hypertrophy	YES (Table 5)	Not tested
Altered foci	YES (Table 5)	Not tested
Adenomas or carcinomas	**YES** (see discussion above)	Not tested
CAR activation *in vivo* transgenic CAR knock-out (KO).	Not tested	n.a.

Other possible modes of action

Overall, nine other possible mechanisms for the formation of liver tumours have been described in the literature. Table 17 outlines these and considers their plausibility with regard to isopyrazam.

Table 17: Consideration of alternative modes of action.

Alternative mode of action	Reason for exclusion
Genotoxicity	Isopyrazam was negative in six *in vitro* investigations of genetic toxicity (see Table 8) and two *in vivo* tests to investigate genetic toxicity (see Table 9).
PPARα receptor activation	Isopyrazam did not increase peroxisomal palmitoyl CoA oxidase or P450 Cyp4a activities in liver peroxisome and microsome preparations respectively (see Table 11)
AhR receptor activation	Isopyrazam did not produce a large increase in P450 Cyp1a EROD activity in liver microsomes (see Table 11)
Estrogenic stimulation	Isopyrazam was not estrogenic in an Oestrogen Receptor transactivation assay *in vitro* (Toole 2011)* or uterotrophic *in vivo* in the ovariectomised rat (Kuhl 2011)*
Statins	Isopyrazam was not designed to inhibit HMG-CoA reductase, nor is there any evidence to suggest that cholesterol levels were increased in rats (Milburn 2007a,c, 2008)*
Cytotoxicity	Isopyrazam did not produce elevations in markers of hepatocyte damage nor was there any evidence of cytotoxicity or regenerative proliferation (Milburn 2007a,b,c,d, 2008, and Shearer 2008)*
Immunosuppression	Isopyrazam did not produce any signs of infection, cytotoxicity and regenerative proliferation in micropathology (Milburn 2007a,b,c,d, 2008, and Shearer 2008)*
Porphyria	Isopyrazam did not produce elevations in hepatocyte damage and regenerative proliferation in micropathology or clinical chemistry (Milburn 2007a,b,c,d, 2008, and Shearer 2008)*
Increased apoptosis	Isopyrazam did not increase rates of apoptosis and regenerative proliferation as determined by micropathology in multiple studies (Milburn 2007a,b,c,d, 2008, and Shearer 2008)*

*Only summaries available to RAC and therefore the robustness of these studies could not be assessed

Remaining uncertainties to the proposed mode of action

The applicant provided a number of mechanistic studies for demonstrating that rat hepatocellular adenoma and carcinomas develop through a phenobarbital-like CAR-mediated mechanism that is widely accepted as not being relevant for humans and that these tumours would not warrant classification for carcinogenicity.

During the Public Consultation, the following uncertainties were raised:

1. Lack of data with CAR-knock out animals

RAC notes this weakness and agrees that it is widely accepted that the indirect measurement of CAR activation through determinations of either mRNA codifying for Cyp2b or EROD activity is a surrogate of CAR activation. However, convincingly demonstrating the role of CAR in cell proliferation and tumour promotion would need further support with CAR-knock out animals and its absence leaves significant uncertainties in the hypothesis that CAR has been responsible for liver tumour formation by isopyrazam.

2. Data with human hepatocytes have been generated with a single donor

It is noted that for isopyrazam the human hepatocytes from one particular donor were receptive to proliferation after exposure to epidermal growth factor. Therefore, the lack of response after exposure to isopyrazam casts doubt on the intrinsic incapability of hepatocytes to proliferate. Testing material from only one donor, even if responding to a positive control, leaves uncertainties with regards to biological donor-to-donor variability. In the PC further data have been submitted on 2 donors and RAC considers that this reduced the uncertainties.

The AOP 41 (Sustained AhR Activation leading to Rodent Liver Tumours) has not been fully ruled out

In this respect, RAC notes that, despite the lack of interaction between isopyrazam and the AhR has not been directly demonstrated, no large activation of Cyp1a was noted; which is a requirement for disproving AhR activation (Peffer *et al*., 2018). Therefore, RAC notes that the activation of AhR is very unlikely because in this case a large increase in EROD activity would have to have been detected.

3. The AOP 32 (Inhibition of iNOS, hepatotoxicity, and regenerative proliferation leading to liver tumours) has not been verified

This AOP has been developed for the only thiamethoxam metabolite that is able to inhibit nitric oxide synthases. Isopyrazam and thiamethoxam have very different molecular structures and therefore it is not to be expected that isopyrazam or its metabolites could inhibit nitric oxide synthases. Overall, RAC notes this uncertainty as of very low concern.

4. The AOP 46 (AFB1: Mutagenic Mode-of-Action leading to Hepatocellular Carcinoma) has not been fully verified.

Aflatoxin B1, after metabolic activation, forms a pro-mutagenic DNA adduct that causes DNA mutations. Isopyrazam was negative in six *in vitro* investigations of genetic toxicity (see Table 8) and two *in vivo* tests to investigate genetic toxicity (see Table 9). Therefore, this AOP is not considered by RAC as a concern.

5. Isopyrazam is a succinate dehydrogenase inhibitor

A concern with succinate dehydrogenase inhibitors has been raised since it is noted that humans with mutations in the succinate dehydrogenase gene can suffer uncontrolled proliferation of cells causing cancer. The tumour formation seems to be a consequence of a long-term accumulation of succinate. In this respect, RAC notes that, according to toxicokinetic information provided in the DAR, the mean terminal elimination half-life is 4.6 hours and bioaccumulation of isopyrazam

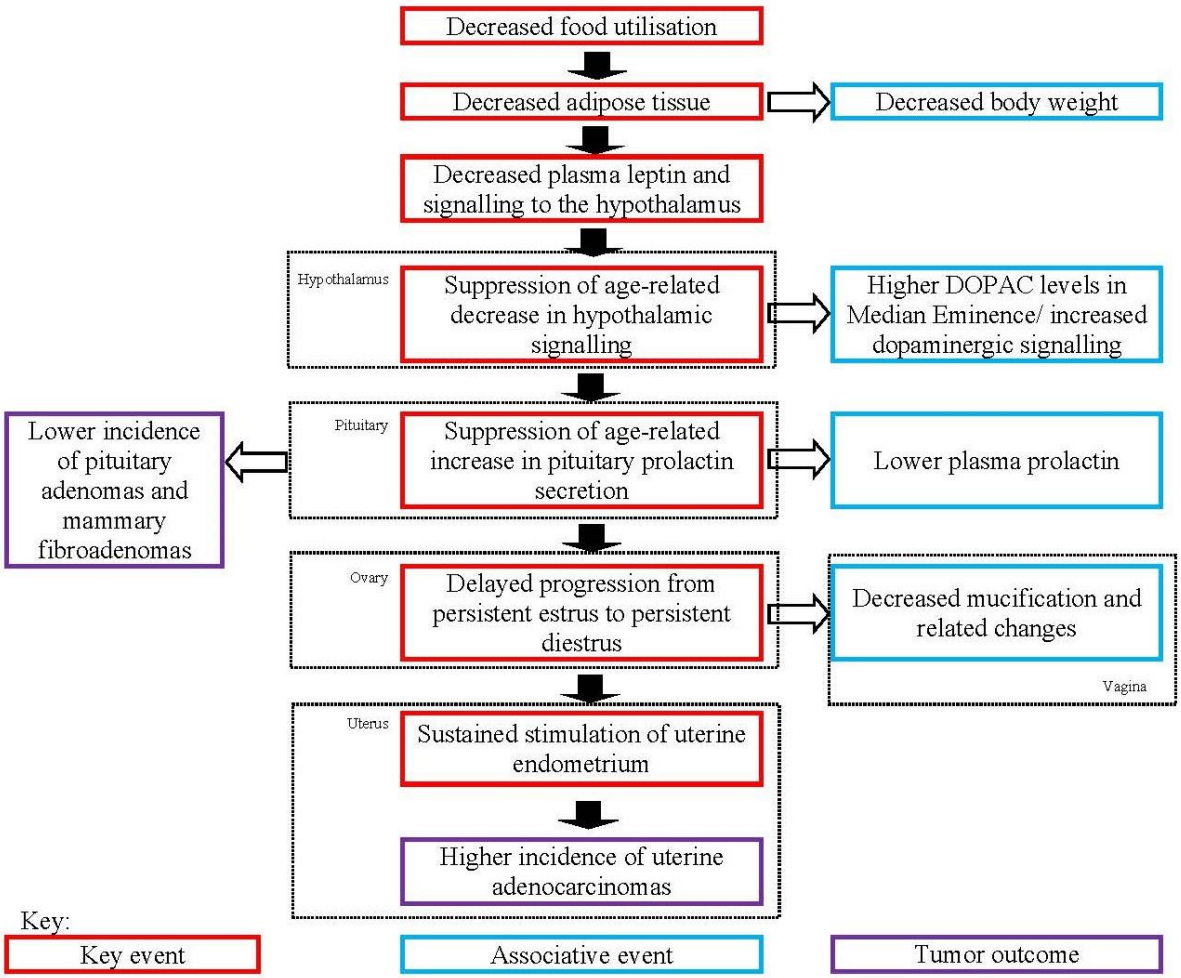
has not been detected. Thus, it is unlikely that isopyrazam exposure could cause succinate accumulation comparable to patients with a genetic disease in which succinate dehydrogenase is not active.

Conclusion on hepatocarcinogenicity

RAC notes that the liver carcinogenicity induced by isopyrazam in rats is consistent with a mode of action based on the CAR activation (Table 16) that is not relevant to humans. However, RAC notes that other possible mechanisms of action have not been sufficiently ruled out and therefore the relevance of the isopyrazam-induced hepatocarcinogenicity for humans has to be considered.

PROPOSED MODE OF ACTION FOR UTERINE ENDOMETRIAL ADENOCARCINOMAS

The incidence of uterine endometrial carcinoma was dose-dependently increased in females, at 3000 ppm, with 29%, exceeding the concurrent control and the available limited laboratory HCD. A delay into reproductive senescence has been proposed by the applicant and DS as the most likely mode of action. The hypothesis is based on the shift in tumour profile observed in high dose rats (increased uterine tumours and decreased mammary and pituitary tumours). The proposed mechanism is described as a biological phenomenon specific for rats, in particular Wistar rats (Harleman *et al*. 2012). The proposed mode of action for the observed shift in the incidence of uterine endometrial adenocarcinomas in the female Han Wistar rat is summarised in the Figure below and was described as follows:



In normal ageing rats (from approximately 12-months) blood levels of prolactin are elevated after the usual dopamine-mediated inhibition of its release is diminished (dopaminergic neurones are lost in the hypothalamus of ageing rats via a process of senescence). Elevated levels of prolactin stimulate continued progesterone synthesis; thus, the normal state of the ageing female rat is high levels of prolactin and progesterone and low levels of oestrogen, follicle stimulating hormone and luteinising hormone. The maintenance of this low oestrogen: progesterone ratio protects against endometrial cancer, owing to a decreased oestrogenic proliferative drive on the uterus; a coincident increase in mammary and pituitary tumours also occurs in the normal ageing rat as a consequence of senescence.

In the isopyrazam treated rats (high dose group) of the two-year carcinogenicity study, along with the increase in the incidence in uterine endometrial adenocarcinomas there was a corresponding decrease in the incidence of mammary gland fibroadenomas and pituitary adenomas. A large sustained decrease in body-weight gain (up to 30-40%) was noted in females at the top-dose, which continued throughout the duration of the study. It is proposed that the decrease in body-weight gain was a consequence of a loss of adipose tissue, which has the potential to cause a delay in the normal age-related loss of tuberoinfundibular dopaminergic (TIDA) neurons of the hypothalamus. As the treated rats age, the retention of a greater number of functional TIDA neurons would result in the continued production of dopamine, thus suppressing prolactin release from the anterior pituitary (via activation of the dopamine-2 receptor). In isopyrazam treated rats, this delay in the physiological age-related increase in prolactin affects the timing of progression into reproductive senescence, subsequently exposing the uterus to a higher oestrogen:progesterone ratio. The result of this continued exposure to higher oestrogen/lower progesterone over time would lead to a pro-proliferative oestrogenic stimulation of the uterine endometrial cells, an increased promotion of spontaneous tumours and ultimately an increase in the observed incidence of uterine endometrial adenocarcinomas. Maintenance of higher dopamine levels also blocks proliferative changes in the pituitary and would account for the lower incidence of pituitary adenoma, whilst the lower prolactin released by the pituitary would explain the observed decline in the incidence of mammary fibroadenomas in the high dose treated groups.

Annex 3 to the CLH report outlines the proposed mode of action and human relevance of the uterine endometrial adenocarcinomas observed in female rats. The key data are summarised below.

Study 1: 18-month investigative study in female rats (Anonymous 2018)

To evaluate the key events of the proposed mode of action, a 18-month investigative toxicity study has been conducted in which female Han Wistar rats were administered isopyrazam for 13, 26, 52, 66 and 80 weeks, the doses reflecting those of the 2-year carcinogenicity study (0, 500 and 3000 ppm equating to 0, 28 and 194 mg/kg bw/day). There were no treatment-related death or clinical signs of toxicity and the results of this study are summarised in Table 18 (for details see CLH report and its Annex):

Table 18: Summary of 18-month study in the rat investigating the mode of action and human relevance of uterine endometrial adenocarcinomas induced by isopyrazam

Control	500 ppm	3000 ppm
↑↑ in prolactin and leptin (age related)	↓ Body weight gain (-9%; last 6-m only) ↓ Final body weight (-5%) ↑ Prolactin (age-related) ↑ Leptin (age-related; week 52)↑ Liver weight	↓ Body weight gain (-32%**) ↓ Final body weight (-21%**) ↓ Absolute fat pad weight (max -59% week 66) ↓ Oestrous cycle duration (from week 29) ↓ Prolactin (sign. at wk 80 compared to control) ↓ Leptin (wk 52-66-80) (age-related increase delayed) - No concomitant alteration in adiponectin (↑) Dopamine (DA) (26% at wk. 26 only) ↑ Dihydroxyphenylacetic acid (DOPAC: 29% at wk 52 and 30% at wk 80) – no clear time course - No change in tyrosine hydroxylase expression - No change in DA/DOPAC turnover ↑ Liver weight

* Statistically significant difference from control group mean, $p<0.05$ (Student's t-test, 2-sided)

** Statistically significant difference from control group mean, $p<0.01$ (Student's t-test, 2-sided)

In the 18-months mechanistic study, body weight gain and adipose fat tissues were significantly reduced at the high dose. Leptin concentration was altered (age-related increase was delayed), but not adiponectin. The extend of the leptin reduction however was consistent with the reduction in adipose tissue (about 60% at week 66). According to the Annex to CLH report, there were no differences between control and treated groups for dopamine concentrations or DOPAC/dopamine ratio (indicative of dopamine turnover). The biological significance of DA increase at week 26 is considered questionable by RAC. The DA/DOPAC ratio was similar across all groups. DOPAC levels tended to be greater than control, but only at week 52 and 80 significant difference was achieved, thus some evidence is available for isopyrazam effects on dopaminergic system. RAC notes that no alteration of TH mRNA or protein expression was detected.
Age-related increase in prolactin was reduced in the high dose group at week 66 and 80, only at 80 weeks levels were significantly different compared to the control group.

Study 2: Evaluation of pituitary prolactin and hypothalamic tyrosine hydroxylase by immunohistochemistry and in situ hybridisation (Anonymous 2015b, tissues from 2-year study)

Pituitary glands from the 2-year rat carcinogenicity study were assessed to explore signalling pathways of the mode of action. Dose dependent higher levels of tyrosine hydroxylase (TH) mRNA, at 500 and 3000 ppm, compared with controls, were reported in the arcuate nucleus alone and in the arcuate nucleus and median eminence. The corresponding protein expression was only slightly increased in the arcuate nucleus alone at 3000 ppm and, for the arcuate nucleus and median eminence, the protein expression was higher at 500 and 3000 ppm, however, with inverted dose-response. No differences in prolactin between control and the two treated groups were noted; which should suggest that the production of prolactin is normal and just its release (dopamine mediated) is affected. It was concluded that isopyrazam is associated with delayed senescence of dopaminergic neurons in the TIDA region of the hypothalamus. RAC notes that no DA/DOPAC data are available for the 2-year carcinogenicity study.

Study 3: Evaluation of hypothalamic tyrosine hydroxylase in control female Wistar rats at 3, 12 or 24 months (Anonymous 2015a, tissues from 90-day and 2-year studies)

Arcuate nucleous and median eminence in the hypothalamus from control animals of 90-day and 2-year rat studies were assessed for quantification of both mRNA and protein tyrosine hydroxylase. Tyrosine hydroxylase proteins were lower for the 2-year animals compared with the

1-year animals; and were also higher in the 1-year animals compared with the 90-day animals. Messenger RNA codifying for tyrosine hydroxylase was lower in the 2-year animals than in the 1-year animals and no statistically significant differences between the 1-year and 90-day animals. In conclusion, mRNA and protein expression of tyrosine hydroxylase decreases progressively with age (from 1- to 2-years) in control rats.

Key event one: body-weight gain deficit and loss of adipose tissue

In the 18-month investigative toxicity study, the postulated initial key event in the mode of action hypothesis was demonstrated (Table 18, Annex 3 to the CLH report) by a reduction in body-weight gain and final body weight when compared with controls (-32%**, $p<0.01$ and -21%**, $p<0.01$, respectively). In the 3000 ppm isopyrazam treated group in this same study a particularly marked reduction in absolute fat pad weight was also noted (-59%** in week 66) (Table 18). In the 2-year rat carcinogenicity study (Table 5, Annex 3 to the CLH report), there was a large deficit in body-weight gain throughout the study in the 3000 ppm treated group, to a magnitude of 40% lower than controls by the end of the study. Hence, in both studies, a marked reduction in food utilisation efficiency was indicated because no significant differences in food consumption were noted. An increase in the incidence of uterine tumours (with a concomitant reduction in the incidence of mammary and pituitary tumours) as a consequence of body-weight loss has been previously demonstrated in rats after dietary restriction (particularly in the Wistar rat). In fact, as a consequence of reductions in body-weight gain, because of calorific restriction, both Sprague Dawley and Wistar rats showed reductions in mammary and pituitary tumours, but only in Wistar rats there was an increase in uterine tumours under such conditions (Harleman *et al.* 2012). RAC notes the decrease in body weight gain and decrease in adipose tissue, however questions that the decreased body weight gain is considered to be a specific initiating event for the isopyrazam induced uterine tumours. Such tumours were not systematically observed as a result of exposure to substances at high doses even if significant reductions in body weight gain were observed. Could the marked decrease in body weight gain have masked a more pronounced tumourigenic effect? It is also noted that in the 2-year study the pituitary and mammary gland tumours are already reduced at 500 ppm, and the uterine tumour incidences are also slightly increased at this dose. A causal relationship for body weight is considered questionable by RAC.

Key event two: decrease in plasma leptin and signalling to the hypothalamus

In accordance with the hypothesised mode of action, body weight/adipose tissue losses should lead to a reduction in plasma leptin and signalling to the hypothalamus. Circulating leptin levels were measured in the 18-month investigative study (Table 18, Annex 3 to the CLH report). The expected age-related increases in plasma leptin were evident in the control and the 500 ppm isopyrazam treated animals from week 52 onwards (statistically significant compared with the day-28 values). In animals of the 3000 ppm group this increase was delayed until week 80 of treatment (at all ages the mean leptin values were numerically smaller than control values with statistical significance being reached at weeks 52, 66 and 80). RAC acknowledges that leptin was indeed decreased at week 52-80 weeks, at levels consistent with the decrease in adipose tissue (about 60% at week 66), however, no concomitant changes in adiponectin were reported (but would be expected). Importantly also, no causality has been demonstrated for leptin and its signalling and downstream events of the postulated Mode of action.

Key event three: Suppression of age-related decrease in dopaminergic signalling

The proposed mode of action postulates that treatment with 3000 ppm isopyrazam preserves dopaminergic signalling in the TIDA neurons of the hypothalamus in comparison with the physiological reduction in signalling which occurs in control animals of the same age. The TIDA neurons' cell bodies are situated in the arcuate nucleus region of the hypothalamus with their

axons extending into the median eminence region. Dopamine is synthesised in the TIDA neurons and released from the median eminence to reach the anterior pituitary.

The age-related decrease in the number and activity of dopaminergic neurons in normal ageing rats has been investigated via measurement of tyrosine hydroxylase (TH); which is the rate limiting step in the synthesis of dopamine. TH mRNA levels at 2-years were statistically significantly lower than those at 90-days and tyrosine hydroxylase protein was statistically significantly lower at 2-years than at 1-year in these control rats, suggesting that tyrosine hydroxylase and therefore dopamine synthesis reduces over time in normal ageing female rats (investigative study 3, Annex 3 to the CLH report).

In the 18-month investigative study, the median eminence of the hypothalamus was isolated to determine the concentrations of dopamine and its metabolite 3,4-dihydrophenylacetic acid (DOPAC). An elevation of mean DOPAC was observed in the 3000 ppm isopyrazam treated group (Table 18, Annex 3 to the CLH report). In this group, DOPAC levels were higher than controls at week 52 (29%* $p<0.05$), week 66 (16%) and week 80 (30%** $p<0.01$); corresponding to the lower leptin values observed at these time-points; DOPAC and dopamine values were also higher than controls at week 26 (31% and 26%* respectively). Dopamine/DOPAC ratio was similar across all groups. RAC takes note of these effects and the time courses observed in the 18-months study: The mean dopamine (DA) concentrations in the median eminence of the hypothalamus were only statistically significantly higher at week 26, and were not affected later, thus biological significance of this isolated finding at week 26 is questionable. DOPAC showed some alterations without clear time course at week 52 and 80. There were no treatment-related alterations in tyrosine hydroxylase (enzyme in dopamine synthesis) staining in the arcuate nucleus nor in the median eminence. Dopamine turnover was not affected in control and treated groups and the DA/DOPAC ratio was similar across all groups. Evidence for clear effects on dopamine synthesis, concentrations and dopamine/DOPAC turnover, thus functional activity of TIDA neurons seem rather weak.

There was a statistically significantly higher level of TH mRNA in the arcuate nucleus region and the combined arcuate nucleus region and median eminence regions than controls in the 2-year carcinogenicity study (investigative study 2, Annex 3 to the CLH report). Hence, it has been concluded that there is a higher capacity for dopamine production in the TIDA neurons of rats treated with 3000 ppm isopyrazam compared with controls, after 2-years of treatment. However, no DA/DOPAC data are available for the 2-year study and RAC takes note of the inconsistencies in TH mRNA and protein expression in the 2-years study (as described above study 2, 2015b). The 18-month and 2-year study results are not in line as there were no treatment-related alterations in TH in the arcuate nucleus nor in the median eminence in the 18-months study.

Despite the uncertainties related to inconsistencies in the results itself, RAC notes that these experiments have research study character and do not follow validated methodologies. They also do not include positive controls or guideline values for evaluation hampering result interpretation.

Key event four: suppression of age-related increase in prolactin

Plasma prolactin levels were measured in the 18-month study in samples taken after 4, 13, 26, 52, 66 and 80 weeks (investigative study 1, Annex 3 to the CLH report). It was demonstrated that an age-related increase in plasma prolactin observed in the control and 500 ppm isopyrazam treated animals was delayed by treatment with 3000 ppm isopyrazam after 66- and 80-weeks' treatment (statistical significance was reached only at week 80). With the delay in the rise in prolactin levels there was a concomitant delay in the onset of reproductive senescence, postulated by the applicant. RAC notes that no prolactin levels are available for the 2-year carcinogenicity study, however mammary gland and pituitary tumours were reduced for mid and high dose. The dose response of the mammary gland tumours in the 2-year study (↓ at 500 and

3000 ppm) is not in line with the dose response for prolactin in the 18-moths study (↑ at 500 ppm, ↓ at 3000 ppm).

Key events five and six: altered transition to reproductive senescence, increased total number of oestrus cycles and proliferation

The 18-months investigative study monitored the pattern and onset of reproductive senescence in female Han Wistar rats as well as the effect of isopyrazam treatment (investigative study 1, Annex 3 to the CLH report). An oestrus cycle is expected to measure between 4 and 5 days in normal sexually mature rats. The four stages that could potentially be observed via vaginal lavage are proestrus (P) during which ovulation occurs, oestrus (E), metestrus (M) and diestrus (D).

All animals showed mean cycle lengths of approximately four days for the first 25 days of treatment and then all showed a tendency for fewer animals with complete cycles and longer mean cycle durations at week 29. Animals treated with 3000 ppm isopyrazam had a greater number of complete oestrus cycles compared with the controls (Table 19). There was a tendency for 3000 ppm isopyrazam treated groups to have shorter mean cycling durations than the other groups (at weeks 77-79 high dose animals had a mean cycle duration of 6.1 days compared with 7.7 and 7.8 days in the control and 500 ppm groups respectively (Table 19)). The control group showed the expected physiological response of females entering reproductive senescence with an initial increase in the percentage of days in an oestrogenic state (between sampling weeks 3-4 and 42-43) (Table 19) followed by a slow decline. In contrast, in 3000 ppm animals, the percentage of days in an oestrogenic state continued to increase up to weeks 55-57 (Table 19) before showing a slow decline. In the 3000 ppm group there was a lower incidence of "reproductive cycle alteration", a term which is assigned when oestrous cycling cannot be determined owing to the onset of reproductive senescence. RAC acknowledges that the data may indicate that isopyrazam has the potential to delay reproductive senescence, probably at 500 and 3000 ppm. However, RAC notes that the GLP statement of the study report mentions that the systems used for calculation and tabulation of estrous cycle data were not validated. The estrogenic state unopposed by progesterone, compared to control group, was postulated, however estrogen:progesterone levels that would lead to a sustained stimulation of the uterine endometrium have not been demonstrated. The demonstration of an alteration of estrus cycle-sensitive estrogen:progesterone ratios would have substantiated such hypothesis. In particular, it is also noted by RAC that across all time intervals, there were no definitive adverse test substance-related histologic changes, and no apparent test substance related effects on proliferative lesions in the uterus, cervix or vagina, which would be indicative of sustained stimulation of the uterine endometrium due to elevated estrogenic/progesterone levels. Thus, the last key event leading to the adverse outcome endometrial adenocarcinoma/hyperplasia, , has not been demonstrated.

Table 19: Summary of findings from the evaluation of oestrus cyclicity.

Parameter	0 ppm (control)	500 ppm isopyrazam	3000 ppm isopyrazam
Oestrus cycle length weeks 0-51 (days)	3.9-4.8	4.0-4.9	4.0-4.7
Oestrus cycle length weeks 52-80 (days)	5.5-7.7	5.3-7.8	4.7-6.1
Animals with complete cycles-weeks 0-25	86.4% - 100%	82.4% - 99.3%	89.6% - 100%
Animals with complete cycles-weeks 29-57	50.5% - 100%	44.2% - 81%	48.6% - 93.3%
Animals with complete cycles-weeks 60-80	37.5% - 44.8%	26.7% - 62.1%	24.2% - 62.5%
Peak oestrogenic state (% E + % P)	59.8%	63.5%	67.3%
Timing of peak oestrogenic state	**42-43 weeks**	50-51 weeks	**55-57 weeks**

Tumour outcome

The pattern of alterations in the incidence of uterine, mammary and pituitary tumours in the 2-year rat carcinogenicity study supports the proposed mode of action hypothesis. Uterine tumours (Table 12) were increased, whilst mammary and pituitary tumours were decreased at the top-dose of 3000 ppm (Table 13). The proposed mode of action and the resulting pattern of alterations in uterine, mammary and pituitary tumours are well described in the literature (Harleman *et al.* 2012). The Han Wistar rat is reported as being particularly susceptible to these changes following significant reductions in body weights; this is demonstrated in the 2-year carcinogenicity study in which the observed shift in tumour profile was related to the magnitude of the effect on body-weight gain.

RAC notes that based on the tumour pattern the hypothesis is plausible, but also notes inconsistencies in the dose-response and time course of the initiating event, key events and the adverse outcome. An example is the change in tumour pattern at mid dose, while body weights and adipose tissue remain fairly unchanged at 500 ppm (as commented above).

Relevance to humans

The CLH report elaborates on the human relevance of the postulated Mode of action. There are fundamental differences between humans and rats with regard to both hormonal control of the hypothalamic-pituitary-gonad axis and the process of transitioning into reproductive senescence. Furthermore, the human reproductive (menstrual) cycle has markedly different control mechanisms compared with the 4-5 day cycle of rats.

The prolactin surge that occurs in rats during proestrus is not observed in humans. In rats, the normal luteal phase is only one day and the new corpora lutea will regress; however, maintenance of a higher prolactin level (i.e. on mating) rescues the corpora lutea and stimulates it to produce further prolactin. In contrast, human corpora lutea are maintained by luteinising hormone during a menstrual cycle and by chorionic gonadotropin from the placenta (in pregnancy). In rats, luteolysis is under the partial control of prolactin whilst luteotrophic actions of prolactin do not occur in humans.

With regard to reproductive senescence processes, in Wistar rats the onset of senescence is driven by a progressive decrease in the activity of TIDA neurons in the hypothalamus and a consequential loss of dopamine-mediated inhibition of prolactin. Prolactin levels are thus elevated resulting in a luteotrophic effect on the corpora lutea, leading to elevated progesterone and lower oestrogen in the blood. This regulation does not occur in humans; in contrast, menopause and reproductive senescence in humans are driven by an eventual depletion (with age) of the limited number of primordial follicles in the ovaries and are associated with a marked decrease in circulating oestrogen and progesterone. The state of persistent oestrus is unique to rats with no equivalent state in humans.

RAC notes as regards to the uterine tumours in general, these tumours are malignant and endometrial cancer is considered highly relevant for women; both in humans and in rodents this type of cancer is an oestrogen sensitive lesion.

Exclusion of other possible mode of action

To support the proposed mode of action, other alternatives have been evaluated in the context of the existing data. Data supporting the exclusion of alternative mode of action is summarised in the Table 20 (see Annex 3 to CLH report for details). In addition, RAC complemented the assessment available in the CLH report:

Table 20: Data supporting the exclusion of alternative mode of action.

Alternative mode of action	Reason for exclusion
Genotoxicity	Isopyrazam was negative in six *in vitro* investigations of genetic toxicity (see Table 8) and two *in vivo* tests to investigate genetic toxicity (see Table 9).
Direct oestrogenicity	Isopyrazam shows no estrogenic potential *in vitro* in an assay with stably transfected human oestrogen receptor-α transcriptional activation* Isopyrazam shows no estrogenic potential *in vivo* in an uterotrophic assay in ovariectomised rats*
Dopamine agonist/effects on the dopamine transporter	Isopyrazam 10 µM (or its metabolite CSCD459488) were not inhibitors of dopamine transport (27% ligand binding) or of dopamine D2 receptor (6% ligand binding) *in vitro** based on competition ligand binding (≥50% inhibition indicates activity). RAC notes uncertainties in the results interpretation as only the result of one single concentration has been presented and no log competition curve of ligand binding, which is usually set up in such type of studies. This is considered important, as the dopamine receptor is a neuro-endocrine receptor and as such operates rather in the nM range. RAC acknowledges further experiments linked to the DA transporter: Isopyrazam is an inhibitor of dopamine transport (uptake into rat striatal synaptosomes) *in vitro* at a concentration of 100 µM* Isopyrazam inhibits control ligand binding to the dopamine transporter protein *in vitro* at a concentration of ≥ 30 µM* Isopyrazam is not an inhibitor of the dopamine transporter *in vivo* assessed based on suppression of a 17β-Estradiol (E2) mediated prolactin surge*
Prolactin secreting tumours in the anterior pituitary	According to the literature, the majority of rat pituitary hyperplasias and adenomas are functional prolactin-producing tumours (Kovacs *et al*. 1977*). No significant differences in the expression of prolactin protein were observed between the control and treated groups in the 2-year rat study. Therefore, the ability of these tumours to secrete prolactin is unlikely to be the cause of the uterine tumours.
Oestrogen metabolism in the uterus	Modulation of oestrogen metabolism in the uterus could potentially lead to higher net oestrogenic stimulation of the uterus and ultimately uterine adenocarcinomas. An example is 17β-estradiol which when hydroxylated to 4-hydroxyestradiol has a stronger carcinogenic potential on the uterus, kidney and pituitary. Cytochrome P450s (predominantly CYP1B1) are responsible for the hydroxylation of 17-β-estradiol. It was demonstrated *in vitro* that isopyrazam elevated levels of CYP2B and CYP3A activity (indirectly measured by associated markers) (Tables 14 and 15); *in vivo* it was shown that isopyrazam increased total hepatic microsomal CYP content (Table 15). The administration of isopyrazam in the diet for 14-days (500 and 3000 ppm) resulted in a significant increase in the metabolism of 17-β-estradiol to 2- and 4- estradiol in liver microsomes, however no changes in uterine CYP1B1 were observed. This suggests that the increase in oestrogen metabolism is a result of CYP2B/3A microsomal enzyme induction, secondary to CAR activation and that peripheral induction of estradiol hydroxylation does not occur in isopyrazam treated rats. Furthermore, the observed shift in tumour profile is inconsistent with this mode of action. Contrary to the reduction in mammary and pituitary tumours observed after isopyrazam exposure (Table 13), such a mode of action would be expected to cause an increase in tumours in the oestrogen sensitive mammary and pituitary tissues. Therefore, the modulation of oestrogen metabolism in the uterus is unlikely to be the cause of the uterine tumours.

Increase in oestrogen:progesterone ratio	Indirect senescent but also chemically-induced imbalance in sex steroid hormones in the ovary leads to decrease of both oestrogen and progesterone and a status similar to a high oestrogen status may manifests by persistent oestrous in vaginal cytology, an atrophic ovary with cystic atretic follicles, lack/few corpora lutea, cornification of the cervix/vagina mucosa, and/or squamous metaplasia of endometrial epithelial cells. Atypical precancerous hyperplasia may be increased (Yoshida *et al*.,2015; Cruz *et al*., 2017). For clarification, no oestrous-cycle-sensitive hormone measurements on oestrogen:progesterone ratio are available. RAC considers that the data available do not allow a firm conclusion on the role of this Mode of action.
Increased *in situ* aromatase	According to Yoshida *et al*. (2015), this is a mechanism with no evidence so far in rodents, but human relevance has been demonstrated with increased protein and mRNA expression for aromatase (and related *in situ* oestrogen production) in epithelial stromal cells in endometrial carcinomas (Watanabe *et al*.1995). Aromatase is a key factor for mammary carcinogenesis. Based on the tumour profile of isopyrazam (tumour shift with decrease in mammary tumours) such pathway is unlikely.
Cytotoxicity / regenerative cell proliferation	Chronic cytotoxicity with subsequent regenerative cell proliferation is considered a mode of action by which tumour development can be enhanced. For isopyrazam no persistent inflammation/ cytotoxicity and regenerative hyperplasia is evident in the repeated dose and carcinogenicity studies. Carcinogenesis due to excessive toxicity and necrosis that would trigger regenerative proliferation is unlikely as there is no histopathological hint for this mechanism. RAC concludes that the data do not support a cytotoxicity-mediated Mode of action.

*Only summaries available to RAC and therefore the robustness of these studies could not be assessed.

Uncertainties in the proposed mode of action

The applicant has provided a number of mechanistic studies for demonstrating that rat uterine tumours are developed through a mode of action based on a delay into the reproductive senescence induced by isopyrazam that is considered non relevant for humans and that would not qualify these tumours for warranting classification for carcinogenicity. Overall, RAC notes the following uncertainties:

- *AOP and previous general considerations of RAC about this particular mode of action*

RAC provided an opinion on about the CLH of sedaxane in 2019. This substance is another 4-carboxamide derivate causing uterine tumours similar to those found in the case of isopyrazam. For sedaxane the mode of action proposed for isopyrazam-induced uterine tumours was also assessed. Some of the concerns raised by RAC were:

It is not considered very plausible, as not all body weight reductions lead to increased tumour formation in carcinogenicity studies, not all chemically induced alterations in reproductive senescence lead to uterine tumour formation, and not all Wistar rat feed restriction studies show an increase in uterine adenocarcinoma. However, it is acknowledged that some studies have shown an association of diet / feeding status with uterine tumour incidences (Tucker et al., 1979; Roe et al., 1995), but the diet composition itself rather than caloric restriction could play a role. Also, an association of caloric/feeding status with (decrease) in mammary gland tumours is known. In the view of RAC it is true that reproduction and oestrous cycling is sensitive to feeding and weight loss (McShane and Wise, 1996; Frisch et al., 1975; Tropp et al., 2001). However, not all studies on caloric restriction that show a delay in reproductive senescence result in induction of uterine carcinoma (Keenan et al., 1995). Then, in the literature it is not well demonstrated

whether activity of TIDA neurons changes during different feeding states and inconsistent results have been reported. Recent findings in transgenic mice suggested that short-term fasting attenuated TIDA neuron activity and increased serum prolactin levels (Kubota et al., 2018).

Moreover, other points highlighted by RAC were:

Based on literature references, the applicant postulated the mode of action involving sustained dopaminergic activity and prolactin-dependent alteration of reproductive senescence with an estrogenic state leading to sustained cell proliferation, the adverse outcome, typically observed with higher frequency in Wistar rats, an increase incidence in uterine adenocarcinoma and concomitant decrease in mammary gland and pituitary tumours. This pathway is discussed by Harleman et al. (2012). The authors point out that such a tumour shift is seen in dietary restriction studies in Wistar rats, but less frequent in SD rats. The prolactin-mediated effect in rats on increased oestradiol:progesterone ratio and resulting uterine tumour formation is considered as not relevant for humans, since prolactin is not luteotrophic in humans. Yoshida et al. (2015) concluded that for such a mode of action extrapolation to humans, more clear evidence is needed.

RAC notes that an AOP in Wistar rats is not so far robustly developed enough with its molecular initiating and key events, and thus, at this stage, the relevance for humans is still uncertain. A putative AOP is under development by U.S. EPA ("*Increased dopaminergic activity leading to endometrial adenocarcinoma (in Wistar rats)*", with reference to Harleman *et al*. 2012 (AOP 112: *MIE: increased dopaminergic activity, → KE: Decrease Prolactin → KE: Increase Estrogen receptor activity → KE: Decrease Progesterone from corpus luteum → KE: Increased Hyperplasia (glandular epithelial cells of endometrium → AO: Increase Endometrial adenocarcinoma*). When considering this AOP in its preliminary stage, not all relevant key events were investigated or demonstrated in the case of isopyrazam (such as estrogen receptor activity, also in relation to decreased progesterone, endometrial hyperplasia). On the contrary other key events have been postulated and investigated but no causal relationship has been proven (such as bodyweight, adipose tissue and leptin decrease). Although RAC acknowledges the efforts of the applicant, the Committee is of the view that the postulated Mode of action remains, to a considerable extend, hypothetical.

- *Experimental methodologies to investigate the AOP and evaluation of results*

When it comes to the experimental tools and methodologies to investigate postulated key events, RAC notes further uncertainties. To robustly demonstrate an AOP, methods should preferably have been reviewed previously and agreed by a recognized authority. Other methods should be well established in the published literature and described regarding their fitness for purpose, direct or indirect (i.e. a surrogate) relation to a key event relevant to the final adverse effect in question, assay repeatability and reproducibility. In the case of isopyrazam, some mechanistic studies have a largely research study character. The interpretation of results is hampered because methodologies are not reviewed/validated, and, for some postulated key events, it is unclear to RAC what should be measured/detected. For other key events it seems that other methods should have been used, such as the measurement of E2/P ratio to proof decreased progesterone. In addition, no studies are included that would provide evidence for causality.

Looking at the results itself, as indicated in the previous text section, RAC notes a range of inconsistencies in dose-responses, time courses, and unclear biological significance of the alterations detected or not detected.

To summarise, during the ECHA Consultation and/or Committee consultation, the following uncertainties were raised:

- *Key event one: body-weight gain deficit and loss of adipose tissue*

RAC notes that a decrease in adipose tissue is a very broad key event, and causal relationship remains difficult to establish. Decreased body weight gain is considered not to be a specific initiating event for uterine tumours, and on the other hand, it needs to be considered whether marked decrease in body weight gain could have masked a more pronounced tumourigenic effect.

- *Key event two: decrease in plasma leptin and signalling to the hypothalamus*

RAC notes that no changes in adiponectin were detected where an increase would have been expected.

- *Key event three: Suppression of age-related decrease in dopaminergic signalling and dopamine turnover*

RAC considers dopaminergic signalling not robustly demonstrated due to inconsistencies in response, time course and dose response of DOPAC, DA and TH. DA level was fairly unchanged, no treatment-related alterations were observed in TH in the arcuate nucleus nor in the median eminence in the 18-months study. Further, DA/DOPAC ratio remained similar across all groups, while DOPAC tended to be greater for the high dose group at weeks 26-80, but significant only at week 52 and 80 with an unclear time course. In contrast, in samples from the 2-year study, TH mRNA and protein levels were increased, but mRNA and protein levels were inconsistently altered for 500 and 3000 ppm dose groups.

- *Key event four: suppression of age-related increase in prolactin*

RAC notes that decrease in prolactin started at 66 weeks. However, there was no DOPAC change at 66 weeks which would have been expected, and further, no data are available on prolactin for the actual 2-years carcinogenicity study.

- *Key events five and six: altered transition to reproductive senescence, increased total number of oestrus cycles and proliferation*

The 18-month isopyrazam study seems to suggest that isopyrazam can delay the time of reproductive senescence onset. It is, however, noteworthy that in the GLP statement of the study report, it is mentioned that the systems used for calculation and tabulation of estrous cycle data were not validated. An alteration of (cycle-sensitive) oestrogen:progesterone ratios should have been demonstrated. There were no definitive adverse test substance-related histologic changes, and no apparent test substance-related effects on proliferative lesions in the uterus, cervix or vagina, indicative of sustained stimulation of the uterine endometrium due to elevated estrogenic/progesterone levels.

Uncertainties are also related to alternative Mode of action.

- *Isopyrazam is a succinate dehydrogenase inhibitor*

See RAC consideration about this issue above in the section about uncertainties for mode of action of liver hepatocarcinomas.

- *Other Mode of action*

RAC considers that the available data on the Mode of action of uterine adenocarcinoma are insufficient to support the -postulated Mode of action by the applicant. A prediction of an alternative Mode of action is not possible and thus the Mode of action remains undefined (see decision tree in Yoshida *et al*., 2015).

Conclusion on uterine adenocarcinomas

RAC notes that, despite the mechanistic studies provided in the CLH report and its Annex 3, a number of questions about the plausibility of the proposed mode of action and its relevance for humans still remain unanswered. Therefore, based on the information available in the CLH report, RAC considers that the increased incidence of uterine adenocarcinoma with a dose response relationship is potentially a treatment related effect and relevant for classification as human relevance cannot be excluded.

Comparison with the criteria

Classification in category 1A concerns substances known to have carcinogenic potential for humans and is largely based on human evidence. Since there are no human data it cannot be concluded that isopyrazam has known carcinogenic potential for humans; therefore Category 1A is not applicable.

Category 1B is for substances where sufficient evidence of carcinogenic potential for humans exist. For that, increases incidences of malignant neoplasms or an appropriate combination of benign and malignant neoplasm must be seen in (a) two or more species of animals or (b) two or more independent studies in one species carried out at different times or in different laboratories or under different protocols. An increased incidence of tumours in both sexes of a single species in a well-conducted study, ideally conducted under GLP, can also provide sufficient evidence. In the case of isopyrazam, the data package contains one study showing increment of malignant (uterine) lesions and hepatocellular adenomas and carcinomas in a single species and therefore the conditions for category 1B are not met.

Category 2 is reserved for substances with evidence of carcinogenicity not sufficiently convincing to place the substance in Category 1A or 1B. It can be set in this category if the evidence of carcinogenicity is restricted to a single experiment, as is the case of isopyrazam.

Overall, based on a weight-of-evidence approach, **RAC proposes classification of isopyrazam as Carc. 2; H351 (suspected of causing cancer).**

RAC evaluation of reproductive toxicity

Summary of the Dossier Submitter's proposal

The reproductive toxicity of isopyrazam has been investigated in a two-generation study in rats and in several developmental toxicity studies in rats and rabbits. The DS proposed no classification for sexual function and fertility and adverse effects on or via lactation based on the lack of effects in the 2-generation study. On the contrary, DS proposed classification of isopyrazam as Repr. 1B (H360D) based on the microphthalmia or "slight small eyes" consistently found in four developmental toxicity studies in rabbits.

Comments received during consultation

One company/manufacturer disagreed with the proposal of classification released by the DS. This company submitted a position paper entitled "*Isopyrazam-Technical Position on the Classification of Isopyrazam for Developmental Toxicity in Rabbits*" with detailed comments. The position paper highlights the following points:

- There is no evidence that isopyrazam causes developmental toxicity in the rat and the only finding of relevance to classification is the observation of microphthalmia and small eyes at high dose levels of isopyrazam (≥600 mg/kg/day) in the rabbit.
- Rabbit studies on isopyrazam were performed by two separate contract research organizations (CRO). The isopyrazam rabbit prenatal developmental toxicity studies were some of the first externally managed studies conducted for Syngenta. Due to differences in ways of working, there were uncertainties in the procedures in place for minimising bias and reporting. Consequently, Syngenta changed CRO during isopyrazam development, which caused a need to change the rabbit strain. Preliminary and definitive prenatal developmental toxicity studies were conducted at WIL Research Laboratories (Ashland, US) in New Zealand White rabbits (WIL 639020 and 639021).
- Two preliminary studies in Himalayan rabbit reported foetal findings of eyes smaller than normal, but not sufficiently small to be described as microphthalmia. Small eyes had not previously been described by RCC CRO company and were not in the laboratory's glossary of foetal effects. In the preliminary study at WIL Research Laboratories in New Zealand Whites, a higher incidence of microphthalmia was noted at 1000 mg/kg/day, which was considered related to administration of isopyrazam. However, the maternal toxicity at this dose level was excessive (severe weight loss and abortion necessitating termination). Increased liver weight and centrilobular hepatocyte hypertrophy was noted in New Zealand White rabbits from 150 mg/kg/day.
- In the definitive regulatory rabbit study, there were no occurrences of malformations outside of the laboratories historical control data range.
- Genetics of albinism predisposes laboratory animals to microphthalmia. Albinism of laboratory strains has been associated with a predisposition to microphthalmia due to the expression patterns of genes, which differ from wild type (pigmented) animals.
- Microphthalmia and anophthalmia is common in laboratory rabbits. Microphthalmia / anophthalmia is the second most common major malformation in New Zealand White rabbits, with a maximum incidence of 1.23% per litter (0.07 ± 0.2%, mean ± SD) from 2007 to 2017. Demonstrating a propensity for this abnormality, Charles River (Ashland, USA) reported 20 cases in 17 litters over the same time period. The minor and variant findings reported in Himalayan rabbits were not considered in their foetal glossary, consequently, there was no historical control data available to contextualise the findings. There are significant differences in the calling criteria for microphthalmia and "small eye".
- Higher incidence of microphthalmia associated with poor clinical condition of the dams. The induction of microphthalmia has been associated with the clinical condition of the dam. Consequently, the interpretation of microphthalmia in rabbits is not clear-cut, and it is vital to consider both the species inherent propensity for the malformation and confounders such as maternal toxicity.
- The microphthalmia / small eyes observed in rabbits administered high dose levels of isopyrazam are not considered a specific or sensitive indicator of developmental toxicity. The increases in microphthalmia / small eyes are considered secondary to enhancement of an underlying physiological process predisposing the animals to this malformation. The litter incidence of microphthalmia in isopyrazam treated groups was only a single incidence higher than the concurrent controls, and, in light of the excessive toxicity observed at this dose level, the microphthalmia and small eyes were considered a secondary consequence of maternal toxicity.

Overall, the company/manufacturer considered that the evidence is not sufficiently clear for Category 1B 'Presumed human reproductive toxicant' (H360D) and considered Category 2 'suspected of damaging the unborn child' (H361d) the only suitable remaining category. DS thanked the comments, commented that they should be taken into consideration by RAC but remained in the opinion that Repr 1B (H360D) is appropriate.

One MSCA supported Repr. 1B H360D but requested more information for substantiating the lack of relevance of the delay in vaginal opening and preputial separation. This MSCA also questioned the validity of the HCD (only 2 studies were available) for the number of implantation sites and mean litter size and demanded more weight for the concurrent control group. The DS thanked the comment, provided the requested information for delayed preputial separation and vaginal opening (also shown below in this opinion) and highlighted the fact that the available HCD is relevant in terms of the timing and supports the conclusion that the findings reflect normal background variation. The DS considered that the HCD should not be disregarded and, on the opposite, should be considered in a weight of evidence approach together with other evidences.

A second MSCA also supported Repr. 1B (H360D) but proposed also H360f based on the fact that total litter size was significantly decreased for both the F1 (-13%* $p<0.05$) and F2 (-12%* $p<0.05$) generations, in comparison with the controls; the number of implantation sites in the F0 and F1 mothers were also significantly decreased (12.3 v.s. 10.7* $p<0.05$ and 12.8 v.s. 11.4* $p<0.05$, in controls and F0 then F1 mothers, respectively) compared to controls and the low reliability of HCD since only 2 studies were available in HCD. DS thanked the comments and replied the same considerations stated above as regard the suitability of the HCD.

Assessment and comparison with the classification criteria

Sexual function and fertility

Table 21 summarises the 2-generation study in rats.

Table 21: Summary of animal studies on adverse effects on sexual function and fertility with isopyrazam.

Method	Results	Reference
Multigeneration study HsdRCCHan:WIST rats OECD 416 GLP Isopyrazam (batch: SMU6AP001) Purity: 96.4% (w/w) **93:7 *syn*:*anti*** 0, 100, 500 and 3000ppm	<table><tr><td></td><td colspan="6">mg/kg bw/day</td></tr><tr><td></td><td colspan="2">100 ppm</td><td colspan="2">500 ppm</td><td colspan="2">3000 ppm</td></tr><tr><td></td><td>M</td><td>F</td><td>M</td><td>F</td><td>M</td><td>F</td></tr><tr><td>F0 premating</td><td>8</td><td>9</td><td>41</td><td>47</td><td>250</td><td>277</td></tr><tr><td>F0 gestation</td><td>-</td><td>7</td><td>-</td><td>37</td><td>-</td><td>217</td></tr><tr><td>F0 lactation</td><td>-</td><td>25</td><td>-</td><td>119</td><td>-</td><td>700</td></tr><tr><td>F1 premating</td><td>9</td><td>10</td><td>48</td><td>50</td><td>288</td><td>301</td></tr><tr><td>F1 gestation</td><td>-</td><td>8</td><td>-</td><td>41</td><td>-</td><td>239</td></tr><tr><td>F1 lactation</td><td>-</td><td>24</td><td>-</td><td>129</td><td>-</td><td>774</td></tr></table> There were no treatment related deaths or clinical signs of toxicity **Parental toxicity** (all effects were statistically significant) 3000 ppm <table><tr><th></th><th colspan="2">F0</th><th colspan="2">F1</th></tr><tr><th></th><th>males</th><th>females</th><th>males</th><th>females</th></tr><tr><td>↓ Body weight (end premating period)</td><td>11%</td><td>8%</td><td>5%</td><td>6%</td></tr><tr><td>↑ Liver weights</td><td>18%</td><td>29%</td><td>27%</td><td>31%</td></tr></table>	Anonymous 2008b

↓ Ovary weight	-	13%		27%
↓ uterus + cervix weight	-	27%		39%
Hepatocellular hypertrophy	Minimal or slight		Minimal or slight	

500ppm

	F0		F1	
	males	**females**	**males**	**females**
↓ Body weight (end premating period)	-	2%	-	8%
↑ Liver weights	-	8%	10%	15%
Hepatocellular hypertrophy	Minimal or slight		Minimal or slight	

100ppm

	F0		F1	
	males	**females**	**males**	**females**
↑ Liver weights	-	-	-	10%

Fertility

F0 generation: No adverse effects

F1 generation: No adverse effects

Offspring toxicity (all effects were statistically significant)

3000 ppm

	F1	F2
Mean litter size at birth (inc dead pups)	10.0 vs 11.5 g (HCD[a]: 11.0/9.6)	11.0 vs 12.5 g (HCD[a]: 10.1/10.9)
Implantation sites	10.7 vs 12.3 (HCD[a]: 11.6/10.3)	11.4 vs 12.8 (HCD[a]: 11.3/11.5)
↓ Mean pup weight males day 29 (weaning)	12%	15%
↓ Mean pup weight females day 29 (weaning)	10%	16%
↑ Liver weights in males	26%	23%
↑ Liver weights in females	26%	32%
↑ thymus weights in females	15%	-
[a] Historical control values, from two previous studies conducted at Central Toxicology Laboratory (2007-2008)		

Parental toxicity

Toxicity was evident in both the F0 and the F1 parental generations (Table 21). At 3000 ppm body weights were lower than controls throughout the entire study. By the end of the premating period, body weights in males and females of the F0 generation were 11% and 8% lower than controls, whilst those of the F1 generation were 5% and 6% lower. Body weights of females at 500 ppm were also lower than controls by the end of pre-mating period (2% and 8% lower in the F0 and F1 generations respectively).

Liver weights were increased in all treated animals of both parental generations (Table 21). Relative liver weights for the F0 generation were 18% and 29% greater than controls in males and females respectively at 3000 ppm, and 8% greater in females at 500 ppm. For the F1 generation the increases were 27% and 31% at the top-dose and 10% and 15% at the mid dose in males and females respectively; liver weights were also increased in females of the F1 generation at 100 ppm (10%). Accompanying histopathological signs were present in both parental generations at 500 and 3000 ppm and comprised a dose-related increase in the incidence of hepatocellular hypertrophy (diffuse at the high dose and centrilobular at the mid dose) with fine cytoplasmic vacuolation apparent in the hypertrophic hepatocytes. RAC notes that these hepatic effects were already noted in the repeated dose toxicity studies and were considered as adaptive. Ovary and uterus weights were increased at the top-dose in both generations. However, in the absence of microscopic findings or functional effects on fertility RAC notes that these findings were unlikely to be treatment related.

Fertility

Isopyrazam exposure had no adverse effects on fertility or reproductive performance in either generation. Oestrous cycling and sperm parameters were unaffected by treatment.

Offspring toxicity

At the top-dose of 3000 ppm, the total litter size was smaller than controls for both the F1 (-13%* $p<0.05$) and F2 (-12%* $p<0.05$) generations; the number of implantation sites in the F0 and F1 mothers were also lower than controls (13%* and 11%* respectively) (Table 21). However, these values were within the range of the laboratory's historical control data (based on two studies conducted with the same strain of rat within 2-years of the current study) and so are most likely to reflect normal background variation. There was no effect on pup appearance, pup survival or sex ratio. Although pup body-weight at birth was not affected by treatment, body-weight gain during lactation was reduced at the top-dose in both generations, such that at weaning body weights were approximately 11% and 16% lower than controls for the F1 and F2 generations respectively.

Similarly, to the parental generations, liver weights of the F1 and F2 offspring killed at weaning were increased by 23-32% (3000 ppm) and 6-14% (500 ppm). Thymus weights were increased in the F1 but not in the F2 females; the lack of consistency across generations suggests that this is a chance finding.

In F1 males at the top-dose, the age of preputial separation was 2.3 days later than controls, whilst in females of this group, vaginal opening occurred 2 days later than controls; however, these observations are likely to be a secondary consequence of the reduced body-weight gain and the subsequent lower post-weaning body-weights that were observed in this dose-group. Table 22 shows further information relating to the preputial separation and vaginal opening.

Table 22. Relationships between days of preputial separation and vaginal opening with mean body weight in F1 generation.

		Dietary concentration			
		0	**100**	**500**	**3000**
No animals		26	26	26	26
Day of preputial separation (mean)		45.0	45.1	45.8	47.3**
	SD	1.7	1.6	2.5	2.4
Mean body Weight at landmark (g)		171.9	170.4	171.0	159.2**
	SD	15.7	14.5	19.5	17.6
Day of vaginal opening (mean)		36.2	36.9	36.6	38.2**
	SD	1.2	1.8	1.8	2.0
Mean body Weight at landmark (g)		105.3	104.5	102.4	103.7
	SD	7.8	11.2	8.4	13.8

* Statistically significant difference from control group mean, $p<0.05$ (Student's t-test, 2-sided)
** Statistically significant difference from control group mean, $p<0.01$ (Student's t-test, 2-sided)

Development

The potential of isopyrazam to adversely affect development has been assessed in rats in one study using the 93:7 *syn*:*anti* isopyrazam and in another using the 70:30 ratio. In rabbits, three dose-range finding studies and one main study are available to assess the developmental toxicity of isopyrazam (all conducted using the 93:7 *syn*:*anti*).

Developmental toxicity study in rats with preparation syn:anti ratio 93:7 (Anonymous 2007)

Isopyrazam (93:7 *syn*:*anti* , purity 96.4%) was administered via gavage to groups of 24 mated HsdRccHan:WIST rats at doses of 0, 20, 75 and 250 mg/kg bw/day, during days 5 to 21 of gestation; day of mating (detection of sperm detected by vaginal smear) was presumed to be gestation day one. Some maternal toxicity was evident at 250 mg/kg bw/d. It was characterised by lower food consumption throughout the study to a magnitude of 43% lower than controls on days 5-8 and 22.5% lower on days 17-20. Maternal body-weight gain was lower than controls throughout dosing (23% lower by day 22), which culminated in mean body weights that were 7% lower than controls on day 22 (4% lower when adjusted for gravid uterine weight). Body-weight loss and poor clinical condition led to the premature death of one female from this dose-group.

Table 23 summarises the caesarean section data. The percentages of pre-implantation losses were statistically significantly higher than those of the controls for all doses. However, it should be noted that pre-implantation losses for the concurrent control (1.9%) was notably lower than the historical control value based on five other studies conducted by the laboratory in 2006 (range 2.7-13% mean 7.5%) and the control animals of a subsequent developmental toxicity study in rats also had a much higher loss of 4.8%. DS concludes that the increases in pre-implantation losses observed in the 250 mg/kg bw/day dose-group are not a consequence of treatment with isopyrazam, especially considering that dosing commenced on gestation day 5, likely after implantation occurred.

A higher percentage of early intra-uterine deaths at 250 mg/kg bw/day (8% (22/273) compared with 1% (3/306) in controls) meant that the value for overall post-implantation losses was statistically significantly higher than concurrent controls for this dose-group (Table 23). Consequently, the number of live foetuses in this dose group was also lower than controls. However, it is again noted that the post-implantation losses in the concurrent control group (1.6%) were considerably lower than the historical control range of 3.2-9.7% (mean 5.5%) based on five other studies conducted in 2006.

A statistically significant lower number of live foetuses in the 75 mg/kg bw/day occurred in the absence of a specific effect on post-implantation loss, therefore it is likely to be a consequence

of the observed pre-implantation losses in this dose-group and thus not related to treatment with isopyrazam.

Table 23: Caesarean section data in the developmental toxicity study in rats with preparation 93:7 *syn*:*anti* (Anonymous 2007)

Observation	Dose (mg/kg/day)			
	0	20	75	250
No. of pregnant dams alive on day 22	23	24	23	22
Mean no. corpora lutea/dam	13.6	13.7	13.0	13.6
Mean no of implantations/dam	13.3	12.5	12.0*	12.4
Mean no. live foetuses/dam	13.1	12.0	11.6*	11.3**
% pre-implantation loss (litter mean)	1.9	8.6**	7.7*	8.1**
% post-implantation loss (litter mean)	1.6	4.2	3.8	8.4**
Mean male foetal weight (g)	4.86	5.04	4.94	4.59**
Mean female foetal weight (g)	4.64	4.74	4.75	4.30**

* Statistically significant difference from control group mean, p<0.05 (Student's t-test, 2-sided)
** Statistically significant difference from control group mean, p<0.01 (Student's t-test, 2-sided)

The overall numbers of foetuses with malformations or variations was not influenced by treatment. However, there were differences from controls in the percentage of treated foetuses with the following specific skeletal variations shown in Table 24. Treatment-related specific skeletal variations at 250 mg/kg bw/day were observed as retardations of ossification of some vertebral centra and arches, as well as in several hind and fore-paw bones. An increase in the number of foetuses with an incomplete xiphoid cartilage of the sternum was also observed at this dose. Some similar effects were also seen at 75 mg/kg bw/d; however, these were only marginally different from controls and are likely to reflect biological variation and are thus not a result of treatment with isopyrazam.

Table 24: Selected specific skeletal variations (number (%) of affected foetuses) in the developmental toxicity study in rats with preparation 93:7 *syn*:*anti*(Anonymous 2007).

Description	Dose (mg/kg/day)			
	0	20	75	250
No. of foetuses examined	301	288	266	249
Cervical centra:				
Centrum 2 not ossified	53 (17.6%)	50 (17.4%)	60 (22.6%)	89** (35.7%)
Centrum 3 not ossified	8 (2.7%)	9 (3.1%)	22** (8.3%)	33** (13.3%)
Centrum 4 not ossified	4 (1.3%)	6 (2.1%)	10 (3.8%)	23** (9.2%)
Centrum 5 not ossified	1 (0.3%)	2 (0.7%)	9** (3.4%)	18** (7%)
Centrum 6 not ossified	2 (0.7%)	2 (0.7%)	5 (1.9%)	14** (5.6%)
Odontoid not ossified	28 (9.3%)	39 (13.5%)	43* (16.2%)	62** (24.9%)
Ventral arch bipartite ossification	85 (28.2%)	64 (22.2%)	62 (23.3%)	38** (15.3%)
Ventral arch fragmented ossification	33 (11.0%)	22 (7.6%)	19 (7.1%)	14* (5.6%)
Sternum:				
Xiphoid cartilage incomplete	15 (5.0%)	23 (8.0%)	25* (9.4%)	31** (12.4%)
Caudal arches:				
Arch 2 incompletely ossified	1 (0.3%)	3 (1.0%)	4 (1.5%)	20** (8.0%)
Arch 2 not ossified	1 (0.3%)	2 (0.7%)	2 (0.8%)	10** (4.0%)
Fore paws:				
Reduced ossification manus	4 (1.3%)	19** (6.6%)	10 (3.8%)	20** (8.0%)
Hind paws:				
Calcaneum ossified	123 (40.9%)	111 (38.5%)	101 (38.0%)	44** (17.7%)
Reduced ossification pes	27 (9.0%)	29 (10.1%)	26 (9.8%)	43** (17.3%)

* Statistically significant difference from control group mean, p<0.05 (Student's t-test, 2-sided)
** Statistically significant difference from control group mean, p<0.01 (Student's t-test, 2-sided)

Developmental toxicity study in rats with preparation 70:30 syn:anti ratio (Anonymous 2008a)

In a second prenatal developmental toxicity study, isopyrazam (70:30 *syn*:*anti* , purity 90.8%) was administered via gavage to groups of 24 presumed-pregnant HsdRccHan:WIST rats. Doses of 0, 20, 75 and 200 mg/kg bw/day were given from gestation days 4 to 20. There were no treatment-related deaths but ventral recumbency and sedation was noted in all dams of the high dose group for the first few days of dosing. Throughout dosing, maternal body-weight gain and food consumption were reduced at 200 and 75 mg/kg bw/d, such that by the end of the study a corrected for gravid uterus body-weight loss of -5.7 g in the high dose group was apparent (compared with a gain of 34.2 g in the controls). In the mid dose group, the maternal, corrected for gravid uterus, body-weight gain was 41% lower than that of the controls.

Isopyrazam had no effect on litter size or on pre- or post-implantation loss; however, mean foetal body-weights were reduced in the 75 mg/kg bw/day (-6%) and 200 mg/kg bw/day (-18%) dose groups (Table 25).

Table 25: Caesarean section data in the Developmental toxicity in rats with preparation 70:30 *syn*:*anti* (Anonymous 2008a).

Observation	Dose (mg/kg/day)			
	0	20	75	200
No. of pregnant dams alive on day 21	23	23	24	24
Mean no. corpora lutea/dam	14.4	14.8	14.4	14.2
Mean no of implantations/dam	13.7	13.9	13.9	13.2
Mean no. live foetuses/dam	12.4	13.1	13.1	12.2
% pre-implantation loss (litter mean)	4.8	6.2	3.5	7.0
% post-implantation loss (litter mean)	9.2	5.9	5.4	7.9
Mean male foetal weight (g)	5.0	4.8	4.7*	4.1**
Mean female foetal weight (g)	4.7	4.6	4.4*	3.9**

* Statistically significant difference from control group mean, $p<0.05$
** Statistically significant difference from control group mean, $p<0.01$

The overall percentage of foetuses with malformations or skeletal variations (excluding ossification) was not influenced by treatment with isopyrazam. The overall incidence of foetuses with visceral variations (50%) was statistically significantly greater than controls (35%) at the high dose. However, the incidence of each specific visceral variation fell within the range of the historical-control data (based on six previous studies conducted over 2006-2007, in the same strain of rat and in the same laboratory) and hence was not a consequence of treatment with isopyrazam.

Slight retardation of ossification was again observed at the high dose of 200 mg/kg bw/day (Table 26). At 200 mg/kg bw/day the incidence of non-ossification of certain vertebral centra and hind limb phalanges was increased, whilst at 75 mg/kg bw/day the incidence of non-ossification of the centrum of one vertebra (the 3rd cervical) was increased. This retardation of ossification is presumed to be related to treatment with isopyrazam.

Table 26: Selected skeletal examination findings in the developmental toxicity in rats with preparation 70:30 *syn*:*anti* (Anonymous 2008a).

Description	Dose (mg/kg/day)			
	0	20	75	200
No. of foetuses examined	136	145	152	141
Cervical centra:				
Centrum 1 not ossified	7 (5%)	13 (9%)	12 (8%)	17 (12%)**
Centrum 2 not ossified	3 (2%)	7 (5%)	9 (6%)	19 (13%)**
Centrum 3 not ossified	3 (2%)	6 (4%)	10 (7)*	13 (9%)**
Hind limb, digit:				
2 proximal phalanx, left not ossified	3 (2%)	5 (3%)	5 (3%)	10 (7%)*
3 proximal phalanx left not ossified	3 (2%)	5 (3%)	3 (2%)	10 (7%)*
4 proximal phalanx left not ossified	3(2%)	5 (3%)	3 (2%)	10 (7%)*
5 proximal phalanx left not ossified	10 (7%)	12 (8%)	14 (9%)	21 (15%)**
5 proximal phalanx right not ossified	10 (7%)	10 (7%)	11 (7)	18 (13%)*

* Statistically significant difference from control group mean, $p<0.05$
** Statistically significant difference from control group mean, $p<0.01$

First dose-range finding developmental toxicity study in rabbits (Anonymous 2008)

In the first dose-range finding study, gavage administration of isopyrazam (93:7 *syn*:*anti* , purity 96.4%) at doses of 0, 100, 200 and 400 mg/kg bw/day isopyrazam (over days 4 to 27 of gestation) caused no observable signs of maternal toxicity in 10/group female Himalayan rabbits. There were no deaths or treatment-related clinical signs of toxicity, body weight and food consumption were not affected and there were no treatment-related macroscopic or necropsy findings.

There was no effect on litter size, pre- or post-implantation losses or foetal body-weights. Foetuses were individually weighed and examined for gross external abnormalities; the heads were separated from half of the foetuses and serially sectioned for internal evaluation (eyes, brain, nasal passages and tongue). At the high dose, the incidence of foetuses with visceral malformations (specifically relating to the cardiovascular system) was greater than controls with altogether seven foetuses from six litters being malformed in this way; however, as these findings were not repeated in several other developmental toxicity studies in rabbits (including those at higher doses) this is not likely to be a treatment-related effect. There was no effect on the number of foetuses with skeletal and/or head malformations/variations.

Two foetuses at 400 mg/kg bw/day presented with eyes of slightly reduced size (approximately 75% of normal size). One of these foetuses also presented with eye retinal folds and narrow choanal. Only limited evidence of a relationship with treatment has been demonstrated in this specific case; however, as eye abnormalities have been observed in subsequent studies in rabbits, a treatment-related effect cannot be ruled out.

Second dose-range finding developmental toxicity study in rabbits (Anonymous 2008c)

In a second dose-range finding study, female Himalayan rabbits (5/group) were administered isopyrazam (93:7 *syn*:*anti* , purity 96.4%) via gavage at dose levels of 0, 600, 800 and 1000 mg/kg bw/day over gestation days 4 to 27. Again, no maternal toxicity was evident at these doses. There were no treatment-related deaths or clinical signs of toxicity. There were no macroscopic or necropsy findings and isopyrazam had no effect on body weight or food consumption.

With regard to developmental toxicity there was no effect on pre- or post-implantation loss or litter size. The following morphological differences comprising 'small eyes' (microphthalmia) or

'slightly small eyes' were noted in isopyrazam treated groups (Table 27); however, there was no clear dose-response relationship.

Table 27: Summary of foetuses (litters) with malformations, variants and eye malformations or variations in the second dose-range finding developmental toxicity study in rabbits (Anonymous 2008c).

Observation	Dose (mg/kg/day)			
	0	600	800	1000
No. foetuses examined macroscopically- external, visceral and skeletal examinations (no. of litters)	33 (5)	27 (5)	23 (5)	32 (5)
No. foetuses with any external and visceral malformation (litters)	1 (1)	4 (2)	0	6 (4)
No. foetuses with eye malformation: small, ~50-75% of normal (litters)	0	2 (1)	0	5* (3)
No. of foetuses with any external and visceral finding (litters)	20 (5)	21 (5)	16 (5)	29 (5)
No. foetuses with eye variant: slightly small, ~75-<100% of normal (litters)	0	9** (3)	5** (2)	10** (3)

* Statistically significant difference from control group mean, $p<0.05$ (Student's t-test, 2-sided)
** Statistically significant difference from control group mean, $p<0.01$ (Student's t-test, 2-sided)

The laboratory has defined 'small eyes' as an abnormality (50% to 75% of normal size) and 'slightly small eyes' as a variant (75% to <100% of normal size). According to the laboratory HCD for this strain of rabbit (based on 5 studies comprising 679 foetuses conducted in 2006-2007) only one animal presented microphthalmia ('small eyes') and the variant 'slightly small eyes' has never been reported for this laboratory. Microscopic examination of foetal head sections revealed intraocular abnormalities in at least those foetuses that presented with 'small' or 'very small eyes' comprising retinal dysplasia, choroidal dysplasia and/or posterior fibre disarray. These findings are consistent with the presence of microphthalmia and thus confirm the occurrence of eye abnormalities in the treated groups.

Third dose-range finding developmental toxicity study in rabbits (Anonymous 2008a)

A third dose-range finding study has been conducted in which doses of 0, 400, 700 and 1000 mg/kg bw/day of isopyrazam (93:7 *syn*:*anti* , purity 96.4%) were administered via gavage to female New Zealand White rabbits (10/group) over gestation days 7 to 28. There was evidence of maternal toxicity in all dose-groups comprising reduced food consumption, body weights and faeces production. This resulted in two females being killed *in extremis* on days 23 (400 mg/kg bw/d) and 21 (1000 mg/kg bw/d) and led to two females aborting on day 25 at 400 and 700 mg/kg bw/day. Initial body-weight losses in all treated groups, up to a magnitude of -97 g in the high dose group, resulted in overall body weight-gains (not corrected with gravid uterus weight) that were 63% and 53% lower than controls in the 700 and 1000 mg/kg bw/day treated groups, respectively. Relative liver weights were increased in treated groups by a magnitude of 39-77% in comparison with controls.

There was no effect on litter size or pre-implantation loss; however, post implantation losses were higher than in controls in all treated groups (albeit without statistical significance) and at 1000 mg/kg bw/day mean foetal body-weights were 14% lower than controls.

Similarly to the previous dose-range finding studies that were conducted in Himalayan rabbits, there was an increase in the incidence of foetuses with microphthalmia at 1000 mg/kg bw/day. The incidence of rabbits presenting eye variations and malformations at 1000 mg/kg bw/day (7%, 5/76 animals) was substantially greater than the range of the HCD for this strain of rabbit of 0-0.9% (total incidence of 4/6125 foetuses from 33 studies). In three of these five cases, microphthalmia was associated with a haemorrhagic ring around the eye (one foetus), reddened eyes (two foetuses) and/or dark red areas of the eye (one foetus). Microphthalmia has been

observed in other developmental toxicity studies in rabbits, therefore the incidences seen in this study are likely to be related to treatment with isopyrazam.

Prenatal developmental toxicity study in rabbits (Anonymous 2008b)

The main prenatal developmental toxicity study was conducted in New Zealand White rabbits. Gavage doses of 0, 30, 150 and 500 mg/kg bw/day isopyrazam (93:7 *syn*:*anti* , purity 96.4%) were administered to 25/group mated females from day 7 to day 28 of gestation. One female at 500 mg/kg bw/day was found dead on day 24 following body-weight losses and reduced food consumption. Food consumption was reduced for this group overall with an associated reduction in faeces production, although maternal body weights were generally not affected by treatment. Liver weights were increased at 150 and 500 mg/kg bw/day (13% and 36% respectively) and associated findings of minimal to mild centrilobular hepatocellular hypertrophy/vacuolation were evident.

Isopyrazam treatment had no effect on litter size or on pre- or post-implantation losses. Mean foetal body-weights were 8% lower than controls at 500 mg/kg/day with statistical significance being reached for males only. Treatment with isopyrazam had no influence on the overall incidence of foetal malformations or variations.

Of the seven foetuses presenting malformations at 500 mg/kg/day, only one had microphthalmia. Although this incidence did not exceed the laboratory HCD range (0-0.9% from 33 studies, overall control incidence 4/6125 foetuses), similar eye malformations have been reported in other isopyrazam rabbit developmental toxicity studies (at higher doses)therefore a relationship with treatment cannot be unequivocally excluded.

Adverse effects on or via lactation

Information relevant to any potential adverse effects on or via lactation after the administration of isopyrazam can be derived from the two-generation study in rats. In this study, it was concluded that no effects on pups during lactation were attributable to isopyrazam administration.

COMPARISON WITH THE CRITERIA

Sexual function and fertility

The 2-generation reproduction study in rats showed slight lower mean litter size at birth and implantation sites in both F1 and F2 (Table 21). However, these values were within the HCD. RAC notes that the HCD package is formed by only two studies. However, these studies are relevant in terms of timing and performing facility. Overall, considering the small magnitude in the reductions of the mean litter size at birth and implantation sites and the HCD in a weight of evidence approach, RAC considers these effects as non-treatment related and therefore not able to support a classification.

The 2-generation reproduction study in rats reported a delay of 2.3 days in preputial separation and a delay of 2.0 days in the vaginal opening of the F1 animals. Table 22 shows that the mean body weight of males at the moment of preputial separation was 7% lower than controls (statistically significant); while the mean body weight of females at the moment of vaginal opening was 2% lower than controls (non-statistically significant). Overall, considering the weight reductions and that the effects appear only in F1 and not in F2, RAC considers vaginal opening and preputial separation delays as incidental and not robust enough for warranting a classification.

According to the CLP criteria, adverse effects on sexual function and fertility include those that interfere with the reproductive system, onset of puberty, gamete production/transport, reproductive cycle, sexual behaviour, fertility, parturition, pregnancy outcome, reproductive senescence or any other function that is dependent on the reproductive system. All of these have been sufficiently investigated in a guideline-compliant two-generation study, conducted in

accordance with OECD TG 416. Exposure to isopyrazam showed no indication of adverse effects on sexual function or fertility according to these criteria. Therefore, RAC supports the DS's proposal for **no classification of isopyrazam for adverse effects on sexual function and fertility.**

Development

Alterations in post-implantation loss and reductions in male and female foetal weight were noted in both developmental toxicity studies in rats (Tables 23 and 25). However, RAC notes that these effects were reported at top doses where severe maternal toxicity (ventral recumbency and sedation for the first days of dosing, poor physical condition warranting sacrifice by day 21 and body weight loss by the end of the study) was concurrent. It is also noted that the incidence of post-implantation loss was just within the range of the laboratory HCD and that the losses observed in the concurrent control group were considerably low. Further, there were no effects on post-implantation loss (or early intrauterine death) in a second study in rats at a slightly reduced top dose of 200 mg/kg bw/day. Overall, these findings are not considered by RAC to support classification for developmental toxicity.

The developmental toxicity studies in rats also showed delayed ossification (cervical centra, sternum, caudal arches, fore paws, hind paws) (Tables 24 and 26) at top maternally toxic doses. However, RAC notes that these effects also appear, mainly in the study with 93:7 *syn*:*anti*) at the non-maternally toxic dose of 75 mg/kg bw/day (Table 24). RAC notes that this delay in ossification can be used for supporting a classification for developmental toxicity at least as Repro 2.

Microphthalmia was observed in rabbits of both the Himalayan and the New Zealand White strains and was not always accompanied by excessive maternal toxicity and even in some cases appeared without any maternal toxicity. Table 28 summarises these malformations.

Table 28: Summary of eye malformations/variations in rabbits.

Study	Strain	Dose levels	Dose-levels where eye effects seen	Maternal toxicity	No animals examined (No of litters)	Incidence of microphthalmia foetus (litter)	Incidence of 'slightly small eyes' foetus (litter)	HCD
Dose-range finding prenatal developmental toxicity study	Himalayan rabbit	0, 100, 200 and 400 mg/kg bw/day	400 mg/kg bw/day	None	57 (9)	0	2(1)	1/679
Dose-range finding prenatal developmental toxicity study	Himalayan rabbit	0, 600, 800 and 1000 mg/kg bw/day	1000 mg/kg bw/day	None	32 (5)	5 (3)*	10 (3)*	1/679
			800 mg/kg bw/day		23 (5)	0	5 (2)**	

			600 mg/kg bw/day		27 (5)	2 (1)	9 (3)**	
Dose-range finding prenatal developmental toxicity study	New Zealand White rabbit	0, 400, 700 and 1000 mg/kg bw/day	1000 mg/kg bw/day	↓ body weight-gain (53%)	76	5(2)		4/6125
Prenatal developmental toxicity study	New Zealand White rabbit	0, 30, 150 and 500 mg/kg bw/day	500 mg/kg bw/day	↓ food consumption/faeces production No effect on body weight	202 (23)	1	0	4/6125

* Statistically significant difference from control group mean, $p<0.05$
** Statistically significant difference from control group mean, $p<0.01$

Two preliminary studies were conducted in this Himalayan rabbits and the relevant historical control data reported microphthalmia at an incidence rate of 1/679. In the first study, two foetuses (from one litter) at 400 mg/kg bw/day and one control animal, presented with the variation 'eyes of slightly reduced size' whilst no cases of microphthalmia ('eyes of reduced size') were reported. No maternal toxicity was evident in this study up to and including the high dose of 400 mg/kg bw/day.

In the second preliminary study in the Himalayan rabbit, 'eyes of slightly reduced size' were reported in 10(3), 5(2) and 9(3) foetuses (litter) in the 1000, 800 and 600 mg/kg bw/day dose-groups. In addition, 5(3) and 2(1) foetuses (litter) at 1000 and 600 mg/kg bw/day presented with the eye malformation microphthalmia ('eyes of reduced size'). No signs of maternal toxicity were evident in this study up to and including the dose at which the eye effects became apparent.

One preliminary and one main study have been conducted in the New Zealand White strain of rabbit; according to the historical control data for this strain, microphthalmia has been reported in 4/6125 foetuses. In the preliminary study, 76 animals were examined and microphthalmia was noted in five foetuses (from two litters). All these cases occurred at the top-dose of 1000 mg/kg bw/day, a dose level that also induced severe maternal toxicity. Such maternal toxicity became evident from 400 mg/kg bw/day and was characterised by mortality, abortions, lower body weight and body-weight gain, reduced food consumption and lower faeces production. Body-weight losses throughout the study at the top-dose of 1000 mg/kg bw/day (up to -97 at weeks 10-13) resulted in overall body-weight gains that were 53% lower than controls by the end of the treatment period. Large reductions in body-weight gain were also seen at the mid dose of 700 mg/kg bw/day (63% lower than controls by the end of the study). Hence, the microphthalmia observed in the New Zealand White rabbits in this preliminary study occurred only in the presence of marked maternal toxicity.

A high dose of 500 mg/kg bw/day was selected for the main study. There were no signs of maternal toxicity at this dose and 1/202 rabbits presented microphthalmia. This was within the range of the HCD for this strain; however, as similar effects were seen across all developmental studies in rabbits, a relationship with treatment cannot be excluded.

In conclusion, RAC notes that there were incidences of microphthalmia or 'slightly small eyes' that were outside the historical control data and could reasonably be considered to be related to treatment with isopyrazam. Such effects only occurred in the presence of marked maternal toxicity in the New Zealand White rabbit (dose-range finding study) whereas in the Himalayan strain of rabbit, no maternal toxicity accompanied the finding. However, it is noted that the studies in the Himalayan rabbit were preliminary studies only and there is a question about the reporting of the finding by the test laboratory (i.e., the test report refers to the finding as 'eyes of slightly reduced size' and 'eyes of reduced size' rather than microphthalmia). That said, the overall conclusion of the test report (which included a microscopic examination of the foetal head sections and consideration of the intraocular abnormalities) was that the findings were consistent with microphthalmia.

Overall, as the same effect on the eyes has been observed in multiple studies and is consistent across two different strains of rabbit, it cannot be confidently dismissed as a secondary effect of maternal toxicity. The effects were supported by other eye effects observed, such as eye retinal folds, retinal dysplasia, choroidal dysplasia, posterior fibre disarray, haemorrhagic ring around the eye, reddened eyes and dark red areas of the eye, supporting that the eye is a target organ. Therefore, RAC, based mainly on these effects on eyes and supported by the delayed ossification in the studies in rats, supports the DS's proposal for **classification of isopyrazam as Repr 1B (H360D: May damage the unborn child).**

Adverse effects on or via lactation

According to the CLP criteria, classification for effects on or via lactation are assigned can be assigned on the: (a) human evidence indicating a hazard to babies during the lactation period; and/or (b) results of one or two generation studies in animals which provide clear evidence of adverse effect in the offspring due to transfer in the milk or adverse effect on the quality of the milk; and/or (c) absorption, metabolism, distribution and excretion studies that indicate the likelihood that the substance is present in potentially toxic levels in breast milk.

No human evidence is available and there is no toxicokinetic information to indicate that toxic concentrations of isopyrazam or its metabolites may be present in milk. In a 2-generation reproductive toxicity study signs of toxicity in the offspring during lactation (reductions in body weight gain) are not attributable to isopyrazam. Therefore, the criteria for classification for effects on or via lactation are not met and RAC supports the DS's proposal for **no classification of isopyrazam for effects on or via lactation.**

Concentration limits

According to ECHA guidance, when the evidence does not indicate which of the ED_{10} (pups or litter) is more appropriate both should be derived and the lower should be used.

Table 28 demonstrated that ED_{10} for pup incidence must be higher than 400 mg/kg bw/day.

The first dose-range finding prenatal developmental toxicity study in Himalayan rabbits showthat the incidence for litters is 11% (1/9) at 400 mg/kg bw/day with no effects at 200 mg/kg bw/day; while the second study in this species show an ED_{10} for litters lower than 600 mg/kg bw/day. The database with New Zealand white rabbit shows no effects at 30, 150 and 400 mg/kg bw/day, with effects starting at 500 mg/kg bw/day. Overall, the ED_{10} for litter incidence is below 400 mg/kg bw/day but above 4 mg/kg bw/day; which suggests that concentration limits for isopyrazam should be set based on the medium potency group. However, the ED_{10} for litter incidence is very close to 400 mg/kg bw/day, and all the other levels are above, which points the weight of evidence towards low potency group.

Furthermore, when considering modifying factors:

- Severity: Microphtalmia should be considered as severe malformation. For these severe malformations a concentration limit lower than the GCL should be set when ED_{10} are close to the lower border of the group. However, RAC notes that, although incidence for litters is lower than 400 mg/kg bw/day the whole database suggests that such ED_{10} is clearly higher above the lower limit of 4 mg/mg bw/day.
- Data availability: RAC notes no issues of concern as regard as this modifying factor.
- Shallow dose-response curve: RAC notes no issues of concern as regard as this modifying factor.
- Mode of action: RAC notes the lack of information on differences in sensitivities between rabbit and human for this effect.
- Toxicokinetics: Considering comprehensive knowledge of all involved toxicokinetic factors RAC notes no information on toxicokinetics suggesting significant differences between rabbits and humans.
- Bio-accumulation: According to CLH dossier, there was no evidence of bioaccumulation potential; however, for the liver and renal fat, steady state was not reached within the 14-day dosing period, so some accumulation potential in these particular tissues over repeated administration cannot be completely ruled out.

Overall, based on the ED_{10} for both litter and foetus incidences and considering modifying factors **RAC considers that isopyrazam should be assigned to the low potency group and proposes the adoption of the SCL (3%).**

RAC evaluation of aspiration toxicity

Summary of the Dossier Submitter's proposal

DS proposed no classification of isopyrazam for aspiration toxicity based on data lacking.

Comments received during consultation

No comments were received during public consultation.

Assessment and comparison with the classification criteria

Comparison with the criteria

RAC notes that the hazard aspiration toxicity is not relevant for solids and therefore supports the **no classification of isopyrazam.**

RAC evaluation of aquatic hazards (acute and chronic)

Summary of the Dossier Submitter's proposal

Interconversion of the enantiomers is unlikely due to structural characteristics of the molecule in relation to the transformations that may occur chemically or enzymatically catalysed under most common environmental conditions. Both of the isomers are considered to be biologically active.

It is noted that acute aquatic toxicity testing with fish based on OECD TG 203 indicates the *anti*-isomer is 5-8 times more ecotoxic than *syn*-isomer (Anonymous, 2007a; Amonymous, 2007b;Anonymous, 2007c, Anonymous, 2007d). As the specification for technical isopyrazam

covers the range of 78-100% *syn*- and 0-15% *anti*-isomer, the available eco-toxicity studies that are using data both on the 70:30 and 90:10 *syn*:*anti* isomer ratios are considered suitable for hazard classification.

The measured water solubility of *syn*-isomer (99.5%) is 1.05 mg/L at 25 °C and pH 7 and of *anti*-isomer (99.6%) 0.55 mg/L at 25 °C and pH 7. Vapour pressure of *syn*-isomer is 2.4 x 10^{-7} Pa and 2.2 x 10^{-8} Pa of *anti*-isomer at 20°C. The Henry's Law Constant for the *syn*-isomer 1.9 x 10^{-4} Pa m^3 mol^{-1} and 3.7 x 10^{-5} Pa m^3 mol^{-1} for the *anti*-isomer indicate the substance is unlikely to be volatile. Based on the physical-chemical information on the isomers isopyrazam is not expected to dissociate.

Degradation

Abiotic degradation

A valid OECD TG 111 study has been presented. The hydrolytic stability of isopyrazam 90:10 *syn*:*anti* (0.32 mg/L, radiochemical purity ⩾96.1%) was studied in sterile aqueous buffer solutions at pH 4, 5, 7 and 9 showing no significant degradation in 30 and 5 days at respective temperatures of 25 and 50 °C.

A valid aquatic photolysis study with isopyrazam (70:30 *syn*:*anti* , radiochemical purity ⩾97%) at a concentration of approx. 0.5 µg/mL has been presented. Sterile buffer or sterile natural water at pH 7 continuously irradiated at 25 °C for up to 29 days under a xenon lamp. Sterile buffer solution DT_{50} values were calculated with ModelManager for the combined dataset of pyrazole and phenyl label tests. The DT_{50} for the study conditions for both labels was 54.3 days with an r^2 value of 0.44. The direct photolysis DT_{50} equates to 61 - 64 days summer sunlight at 30 – 50 °N assuming 12 hour days. Two degradants were observed: CSAA798670 (max. 14.8% AR days 15-21) and CSCC210616 (max. 7.4% AR day 15). Faster indirect photolysis (due to decontaminants in the natural water test system) was observed in the sterile natural water samples with equated DT_{50} of 5.2 - 5.9 days summer sunlight at 30 – 50 °N assuming 12 hour days. Peak concentration of CSAA789670 and CSCC210616 was 36.4% and 20.1% AR respectively at the end of the study (25 days).

Biodegradation

A valid OECD TG 301F manometric respirometry test (Seyfried, 2006) run above the water solubility at ~100 mg/L of isopyrazam (*syn*:*anti* isomer ratio 65.6:27.3; purity 92.9%) under aerobic conditions in activated sludge observed no degradation within 28 days compared to controls and the substance was considered to be 'not readily biodegradable'.

A valid OECD TG 308 study (Stoll and Nicollier, 2008) measuring the metabolism and rate of degradation of isopyrazam (*syn*:*anti* isomer ratio 91.3:8.7; radiochemical purity ⩾96%) under aerobic and anaerobic laboratory conditions in aquatic systems is given. Samples of untreated Rhein river water and Rothenfluh natural pond water and their associated sediments (dry weight) ratios approximately 4:1 w/w for the river system and 5:1 w/w for the pond system were equilibrated in flasks for 4 weeks. Flasks with a nominal initial concentration of 0.04 µg/ml of the radiolabelled substance were incubated at 20±2 °C in the dark for up to 181 days in the aerobic and similarly in anaerobic test systems for 360 days. In all test systems the overall recovery radioactivity ranged from 90.2% to 98.5% of the applied radioactivity (AR).

It has been noted that isopyrazam dissipated from the water phase in both systems with ~15% remaining in aerobic and ~25% in anaerobic system by day 14. Partitioning to sediment with ~69% in aerobic and ~62% in anaerobic sediment was also observed. Minimal mineralisation was observed over the study with <1% AR CO_2 observed in both aerobic and anaerobic test systems.

The estimations were calculated using ModelManager and FOCUS. The more accurate DT_{50} values of 1.2 to 1.5 days were calculated for dissipation from the water phase in aerobic systems using first order multi compartment kinetics as water to sediment dissipation rates did not appear to conform to single first order kinetics. No sediment dissipation rates were calculated.

Low levels of degradants (<5% AR) were observed in both aerobic and anaerobic test systems with two identified (CSCD626401 and CSCD459488).

The river system aerobic and anaerobic DT_{50} were 2 and 8.8 days. The pond system aerobic and anaerobic DT_{50} were 2.6 and 7.1 days respectively. Total system DT_{50} was >> 1 year for both aerobic and anaerobic test systems due to slow degradation in the sediment.

Isopyrazam is considered not readily biodegradable and rapidly dissipating from water to sediment with minimal mineralisation in aerobic and anaerobic conditions. Also the DS considered isopyrazam not rapidly degradable for the purpose of classification due to the substance being hydrolytically stable at environmentally relevant pH and temperature; it is susceptible to photodegradation, however it is noted that the actual degree of photodegradation in the aquatic environment depends on local conditions and seasons and is difficult to quantify and there is insufficient data to assess it. Based on available information the degradation products of the substance are not considered more acutely toxic than the parent substance.

Bioaccumulation

Experimental $logK_{ow}$ values of the *syn*- and *anti*-isomers of isopyrazam are 4.1 and 4.4 respectively at 25 °C and neutral pH.

A valid OECD TG 305 aquatic accumulation and elimination study (Anonymous, 2007) for radiolabelled isopyrazam (70:30 *syn*:*anti*) is available. It is additionally noted the study is considered valid despite only one test concentration (nominally 0.3 µg/l - 1% of the acute fish LC_{50}) instead of two was employed – the factor of 10 lower value was below the limit of detection.

Exposure in a flow-through system to Bluegill sunfish (*Lepomis macrochirus*) ran for 28 days followed by a 14 day depuration period. Mean measured concentrations (97% of the nominal value) were maintained consistently during the exposure period. The mean lipid content of fish on days 0, 42 and during steady state was 6.0, 5.0 and 5.9% w/w respectively. The overall mean lipid content was 5.6% w/w and did not differ from the start by more than ±25%. During the 14 day depuration phase, the levels of [14C] isopyrazam equivalents in the whole fish decreased rapidly. The calculated DT_{90} was 1.15 days.

The calculated kinetic bioconcentration factor (BCF_k) was 406 l/kg; the steady state $BCF_{(whole\ fish)}$ was 441 L/kg. The lipid content at the end of the study was 5.9%. A lipid normalised to 5% whole fish BCF reflecting this would be 374 l/kg based on 14C-residues.

The dossier submitter concluded that although the individual $logK_{ow}$ values of the *syn* and *anti*-isomers are above 4, the experimental BCF values of the substance are below the CLP threshold of 500 L/kg, therefore isopyrazam is **not** considered to meet the CLP criteria for **bioaccumulation**.

Aquatic toxicity

A summary of available valid information on the aquatic toxicity of isopyrazam is presented in the following Tables. A summary of valid information for degradants with regards to their eco-toxicity is also given as supplemental information. However, it is noted that based on available data the degradation products are not considered more acutely toxic than the parent substance and are not taken into account for classification purposes.

Acute toxicity

Table 29: Summary of relevant information on acute aquatic toxicity.

Study	Species	Endpoint	Exposure		Results		Reference
			Design	Duration	Edpoint	Toxicity (mg/L)	
Fish							
Acute toxicity to fish, OECD TG 203, GLP, purity: isopyrazam 92.9% (65.6% syn, 27.3% anti)	Oncorhynchus mykiss (Rainbow trout)	Mortality	Flow-through	96 hours	LC50	0.066 (mm)	Anonymous 2007a
Acute toxicity to fish, OECD TG 203, GLP, purity: isopyrazam 99.7% (89.6% syn, 10.1% anti)	Oncorhynchus mykiss (Rainbow trout)	Mortality	Flow-through	96 hours	LC50	0.063 (mm)	Anonymous 2005a
Acute toxicity to fish, OECD TG 203, GLP, purity: isopyrazam 92.9% (65.6% syn, 27.3% anti)	Lepomis macrochirus (Bluegill sunfish)	Mortality	Flow-through	96 hours	LC50	0.181 (mm)	Anonymous 2007b
Acute toxicity to fish, OECD TG 203, GLP, purity: isopyrazam 92.9% (65.6% syn, 27.3% anti)	Cyprinus carpio (Common carp)	Mortality	Flow-through	96 hours	LC50	0.0258 (mm)	Anonymous 2007c
Acute toxicity to fish, OECD TG 203, GLP, purity: isopyrazam 92.9%	Pimephales promelas (Fathead minnow)	Mortality	Flow-through	96 hours	LC50	0.0263 (mm)	Anonymous 2007d

(65.6% syn, 27.3% anti)							
Acute toxicity to fish, OECD TG 203, GLP, purity: isopyrazam 92.9% (65.6% syn, 27.3% anti)	Danio rerio (Zebrafish)	Mortality	Flow-through	96 hours	LC50	0.3 (mm)	Anonymous 2007e
Acute toxicity to fish, OECD TG 203, GLP, purity: isopyrazam 99.7% (89.6% syn, 10.1% anti)	Pimephales promelas (Fathead minnow)	Mortality	Flow-through	96 hours	LC50	0.034 (mm)	Anonymous 2006
Acute toxicity to fish, OECD TG 203, GLP, purity: isopyrazam 92.9% (65.6% syn, 27.3% anti)	Cyprinodon variegatus (Sheepshead minnow)	Mortality	Flow-through	96 hours	LC50	0.314 (mm)	Anonymous 2007
Acute toxicity to fish, OECD TG 203, GLP, purity: SYN534969 syn isomer 98.9%	Oncorhynchus mykiss (Rainbow trout)	Mortality	Flow-through	96 hours	LC50	0.0469 (mm)	Anonymous 2007a
Acute toxicity to fish, OECD TG 203, GLP, purity: SYN534968 anti isomer 98.6%	Oncorhynchus mykiss (Rainbow trout)	Mortality	Flow-through	96 hours	LC50	0.0092 (mm)*	Anonymous 2007b
Acute toxicity to fish, OECD TG 203, GLP,	Pimephales promelas	Mortality	Flow-through	96 hours	LC50	0.0817 (mm)	Anonymous, 2007c

purity: SYN534969 syn isomer 99%	(Fathead minnow)						
Acute toxicity to fish, OECD TG 203, GLP, purity: SYN534968 anti isomer 98.6%	Pimephales promelas (Fathead minnow)	Mortality	Flow-through	96 hours	LC50	0.0107 (mm)*	Anonymous, 2007d
Invertebrates							
Daphnia sp Acute Immobilisation OECD TG 202, GLP, purity: isopyrazam 92.9% (65.6% syn, 27.3% anti)	Daphnia magna	Acute immobilis-ation	Static	48 hours	EC50	0.044 (n) see study details below	Benyon and Richardson, 2007
Daphnia sp Acute Immobilisation OECD TG 202, GLP, purity: isopyrazam 99.7% (86.9% syn, 10.1% anti)	Daphnia magna	Acute immobilis-ation	Static	48 hours	EC50	0.13 (mm)	Benyon and Ramsay, 2007
Daphnia sp Acute Immobilisation OECD TG 202, GLP, purity: isopyrazam 99.8% (94.9% syn, 4.93% anti) Guideline adapted for the other	Coenagrionidae Crangonyx pseudogracilis Asellus aquaticus Chaoborus sp. Planariidae Cloeon sp. Ostracoda Lymnaea sp.	Acute immobilis-ation	Static	48 hours 24 hours	EC50	>1 (n) >0.74 (mm) >0.775 (mm) >0.730 (mm) >0.750 (mm)	Ashwell and Langridge, 2007

species mentioned	Lumbriculus variegatus Brachionus calyciflorus					>1 (n) >1 (n) >0.9 (mm) >0.955 (mm) >1 (n)	
Algae/other aquatic organisms							
Freshwater Algal Growth Inhibition OECD TG 201, GLP, purity: isopyrazam 99.7% (89.6% syn, 10.1% anti)	Pseudo-kirchneriella subcapitata*	Cell multipli-cation inhibition	Static	72 hours 96 hours	ErC50 ErC50	>4 (mm) >4 (mm)	Volz, 2005
Lemna sp. Growth Inhibition Test OECD TG 221, GLP, purity: isopyrazam (89.6% syn, 10.1% anti)	Lemna gibba	Growth	Static	7 days	ErC50	>0.5 (n)	Everett et al., 2007

Notes:
** value not considered for classification, because based solely on anti-isomer at 98.6% purity*
mm refers to mean measured concentrations
n refers to nominal concentrations
**formerly Selenastrum capricornutum*
Bold value indicates most sensitive acute endpoint relevant to hazard classification proposal

Acute toxicity data is available for fish, invertebrates, algae and aquatic plants using isopyrazam as well as the individual *syn*- and *anti*-isomers.

Twelve OECD TG 203 **fish** acute toxicity are presented. Eight of the studies were conducted with isopyrazam as either with 70:30 or 90:10 *syn*:*anti* isomer ratio; the remaining four with either the *syn*- or *anti*-isomers. Six different fish species of fish (Rainbow trout, Bluegill sunfish, Common carp, Fathead minnow, Zebrafish and Sheepshead minnow) were exposed under flow-through conditions for 96 h. The LC_{50} values ranged from 0.0092 mg/L to 0.314 mg/L. The results are based on mortality observations and mean measured concentrations.

While results indicate the *anti*-isomer is more ecotoxic to fish, it is noted that isopyrazam contains up to 15% of the *anti*-isomer. In addition as acute endpoints using isopyrazam (*syn*:*anti*) for *Oncorhynchus mykiss*, *Pimephales promelas* and *Cyprinus carpio* are similar and acute endpoints

for the *anti* isomer with *Oncorhynchus mykiss* and *Pimephales promelas* are similar, it is unlikely this is due to significant species sensitivity. This is supported by overlapping 95% confidence intervals for 96-hour LC_{50} values. On this basis, it is considered that the LC_{50} for the *anti* isomer is overly conservative and endpoints based on isopyrazam as a mixture of *syn*:*anti* isomers covering multiple fish species are more appropriate for hazard classification.

The DS therefore concluded that *Cyprinus carpio* flow-through study (Anonymous 2007c) with isopyrazam (70:30 *syn*:*anti*) should be used for classification. Nominal exposure ranges are 5.12, 11.3, 24.8, 54.5 and 120 µg/L. Exposure solutions were prepared with the aid of the solvent THF and a solvent control was included. Study conditions were considered acceptable. Measured concentrations were 89 to 108% nominal over the study period and results were based on mean measured concentrations. The 96-h LC_{50} was 0.0258 mg/L (95% confidence intervals 0.0108 to 0.0528 mg/L). There are 4 more studies with isopyrazam at 70:30 or 90:10 *syn*:*anti* ratio in the same range of toxicity (0.0263-0.066 mg/L) for classification purposes supporting the study selected by DS.

The acute toxicity of isopyrazam to invertebrates was studied in valid OECD 202 flow-through tests. Two tests were with water flea and one study included a wide range of non-standard invertebrates despite this the study appears to be valid. The studies were conducted with isopyrazam as either 70:30, 90:10 or 95:5 *syn*:*anti* ratios. EC_{50} value of one of the tests (Benyon and Richardson, 2007) was nominal, it was further noted that an EC_{50} based on mean measured concentrations would be above the chosen sensitive value and would not impact the classification. For the other two the results are given as mean measured concentrations. For the study with the range of non-standard invertebrates (Ashwell and Langridge, 2007) it was noted that mean measured concentrations were not within >120% of nominal for *Cloeon sp.* and *Ostracoda*. However, 30% and 0% effects were observed for each of these species and they are not the most sensitive endpoints for hazard classification so the update of endpoints based on mean measured concentrations was not done. The *Brachionus calyciflorus* species involved a 24 hour test resulting in a 24-h EC_{50} of >1 mg/L based on nominal concentrations as analytical verification was not undertaken.

One valid study each conducted with isopyrazam as 90:10 *syn*:*anti* isomer ratio for algae and aquatic plants is available. The study with *Pseudokirchneriella subcapitata* reported 72 and 96 hour endpoints based on mean measured concentrations E_rC_{50} >4 mg/L respectively.

As the geometric mean measured concentration of the highest test concentration was 83% of nominal the semi-static 7-day toxicity to *Lemna gibba* study used nominal concentrations for reporting E_rC_{50} >0.5 mg/L for frond number and dry weight parameters.

Overall, the DS proposed to use the lowest 96-hour LC_{50} for *Cyprinus carpio* of 0.0258 mg/L based on the study with 70:30 *syn*:*anti* ratio to classify isopyrazam in the Aquatic Acute 1 category with M-factor 10.

Chronic toxicity

Table 30: Summary of relevant information on chronic aquatic toxicity.

Study	Species	Results	Remarks	Reference
Fish Early-Life Stage toxicity, OECD TG 210, GLP, purity 98.9%, GLP, purity: isopyrazam 92.9% (65.6% syn, 27.3% anti)	*Pimephales promelas* (Fathead minnow)	32-day NOEC 0.00287 mg/L (mm) [fry survival]	Valid	Anonymous, 2007f

Daphnia magna Reproduction OECD TG 211, GLP, purity: isopyrazam 92.9% (65.6% syn, 27.3% anti)	*Daphnia magna*	21-day NOEC [reproduction] 0.013 mg/L (n verified)	Valid	Bätscher, 2007
Freshwater Algal Growth Inhibition, OECD TG 201, GLP, purity: isopyrazam 99.7% (89.6% syn, 10.1% anti)	*Pseudo-kirchneriella subcapitata**	72-hour NOErC 0.31 (mm) 96-hour NOErC 0.31 (mm)	Valid	Volz, 2005
Lemna sp. Growth Inhibition Test, OECD TG 221, GLP, purity: isopyrazam (89.6% syn, 10.1% anti)	*Lemna gibba*	7-day NOErC 0.5 (n)	Valid	Everett et al., 2007

Notes:
mm refers to mean measured concentrations
n refers to nominal concentrations
**formerly Selenastrum capricornutum*
Bold value indicates most sensitive chronic endpoint

Chronic toxicity data is available for fish, invertebrates, algae and aquatic plants.

A valid flow-through OECD TG 210 early life stage toxicity *Pimephales promelas* fish study (Anonymous, 2007f) using isopyrazam (70:30 *syn*:*anti* ratio) running for 32 days reflecting 28 days post hatch is presented. Hatch time and rate, development rate, survival and growth (length and dry weight) were recorded: The nominal exposure range was 0.7, 1.5, 3.3, 7.3 and 16 µg/L. Measured concentrations did not remain within 20% of nominal over the study period and ranged from 57 to 113% of nominal so endpoints were determined based on mean measured concentrations; The resulted 32-day NOEC was 0.00287 mg/L.

A valid OECD TG 211 semi-static chronic toxicity to *Daphnia magna* study (Bätscher, 2007) using isopyrazam (70:30 *syn*:*anti* ratio) is available. The 21-day NOEC for reproduction was 0.013 mg/L based on verified nominal concentrations.

A valid study (Volz, 2005) with *Pseudokirchneriella subcapitata* with *syn*:*anti* ratio 89.6:10.1 reported 72 and 96 hour growth inhibition chronic endpoints based on mean measured concentrations NOE_rC 0.31 mg/L respectively.

The 7-day nominal chronic endpoints for toxicity to *Lemna gibba* study (Everett et al, 2007) using isopyrazam at 90:10 *syn*:*anti* ratio were reported as NOE_rC 0.5 mg/L for frond number and dry weight parameters.

It is noted by the dossier submitter that chronic ecotoxicity studies with *Chironomus riparius* are also available employing water-sediment test systems. Given that isopyrazam concentrations declined in the aqueous phases and partitioned to sediment it is unclear if a contribution of the toxicity in this study was due to sediment contact/ingestion. DS believes reliable data is available from standard aquatic test species/test systems which are more sensitive and considered preferable for hazard classification and thus has not included these results into the CLH report.

The study (Anonymous, 2007f) reporting a 32-day NOEC of 0.00287 mg/L (mean measured) based on fry survival which is also considered the lowest value suitable for hazard classification as Aquatic Chronic 1 with M-factor 10.

Comments received during consultation

Four MSCAs supported the proposed environmental classification Aquatic acute 1, H400 (M=10) and Aquatic Chronic 1, (M=10) based on the available data for the most sensitive species (Fish: *Cyprinus carpio*; 96h-LC50=0.0258 mg/L and *Pimephales promelas* 32-day NOEC=0.00287 mg/L), respectively.

Additional information on the toxicity of a degradation product "metabolite CSCD465008" was given regarding a OECD TG 201 test with *Pseudokirchneriella subcapitata* showing E_yC_{50} (72 hours) = 22.44 mg/L, E_rC_{50} (72 hours) = 26.52 mg/L and NOEC (72 hours) = 18 mg/L. This information was noted by the DS.

One MSCA specifically agreed on the approach taken for classification to exclude the studies (Anonymous, 2007b and Anonymous, 2007d) on the *anti*-isomer and only include studies with a representative mixture of the two isomers; it was acknowledged that toxicity testing with fish indicates that the *anti*-isomer may be more ecotoxic than the *syn*-isomer and that isopyrazam contains a mixture of two designated *syn* and *anti*-isomers both considered to be biologically active. This comment was welcomed by the DS.

Another MSCA pointed out the clear difference in toxicity in fish observed between the *syn* and *anti*-isomer where the latter showed higher acute toxicity (1 order of magnitude), also reflected in the outcomes of the 90:10 versus 70:30 *syn*:*anti* isomer ratios. The same trend is also mentioned to be observed in the *Daphnia magna* studies performed with both ratios. It was noted that studies with algae were only performed with 90:10 *syn*:*anti* ratio and results might not reflect the true toxicity. The DS agreed with the above and also pointed out the lack of 90:10 *syn*:*anti* ratio data for the most sensitive species on chronic endpoints. Additionally the DS noted that the application of the order of magnitude observed with fish/invertebrates determined with the 70:30 ratio to the available algal endpoints based on 90:10 ratio, would not impact the classification as they are not lower. Some editorial or/and minor comments were also presented.

Assessment and comparison with the classification criteria

RAC agrees with the DS that isopyrazam can be considered hydrolytically stable in the environment and susceptible to photodegradation.

RAC notes that the degradation data indicates the substance should be considered not readily biodegradable (0% of mineralisation in Seyfried, 2006) and rapidly dissipating to the sediment from water compartment and remaining persistent in the sediment compartment. This is based on the water/sediment simulation study (Stoll and Nicollier, 2008) in aerobic and anaerobic test systems showing total system DT_{50} at 20 °C >>1 year supported by the results of the microcosm study in the aquatic environment (Kuet and France, 2008) with a total system DT_{50} of 21.2 days under natural outdoor conditions.

RAC takes into account the note on the available data showing the degradation products are not considered more toxic than the parent substance and agrees with the DS to not take into account the information for classification purposes.

Overall, isopyrazam is not considered to be rapidly degradable for the purpose of classification according to the CLP criteria.

RAC agrees with the DS and concludes that isopyrazam has low bioaccumulation potential based on the available bioconcentration study in Bluegill sunfish showing a $BCF_{(whole\ fish)}$ value of 441 L/kg despite the information on the partition coefficients of individual *syn*- and *anti*-isomers (Log Kow values of 4.1 and 4.4 respectively).

RAC agrees with the DS that algae toxicity is not expected to be lower than acute and chronic fish endpoints and would not impact the classification if 70:30 isomer ratio is applied to the available algal endpoints determined with the 90:10 ratio.

The presented acute and chronic algal endpoints were determined with the 90:10 *syn*:*anti* ratio (72-h ErC50 >4 (mm) mg/L and NOErC 0.31 (mm) mg/L) whereas for fish were 96-h LC50 0.0258 (mm) mg/L and 32-day NOEC 0.00287 (mm) mg/L and for invertebrate 48-h EC50 0.044 (n) and 21-d NOEC 0.013 (n-verified) mg/L with 70:30 isomer ratio.

RAC agrees with the DS that based on the available data the *anti*-isomer appears to be more acutely toxic to fish and invertebrates than the *syn*-isomer. As for the chronic toxicity test data is available only for 70:30 *syn*:*anti* ratio it is not possible to conclude on the chronic toxicity of the individual isomers.

RAC notes the composition of isopyrazam ranging from 78-100% *syn*- and 0-15% *anti*-isomer and agrees with the DS to base the classification of isopyrazam on the available test data using 70:30 and 90:10 *syn*:*anti* isomer ratios. RAC also notes that the unlikely specie sensitivity based on the test data presented being in the same order of magnitude and the high confidence interval of LC_{50} values (95%) for the substance itself as for the *syn*- and *anti*-isomers individually.

In conclusion, RAC agrees with the DS that the most sensitive fish LC_{50} for *Cyprinus carpio* of 0.0258 mg/L results in a classification of **Aquatic Acute 1, H401** with **M = 10** (considering 0.01 mg/L < LC50 < 0.1 mg/L).

RAC agrees with the DS that the lowest chronic endpoint of 32-day NOEC for *Pimephales promelas* 0.00287 mg/L results in a classification of **Aquatic Chronic 1, H410 with M = 10** as a **not rapidly degradable** substance (considering 0.001 mg/L < NOEC < 0.01 mg/L for non-rapidly degradable substances).

RAC notes if additional data becomes available either on the bioaccumulation potential, the toxicity of the degradation products in the environment and acute or chronic toxicity of isopyrazam or its isomers, the classification could be reconsidered.

Additional references

Yoshida *et al*., 2015, Predictive modes of action of pesticides in uterine adenocarcinoma development in rats Toxicol Pathol 2015; 28: 207–216

ANNEXES:

Annex 1 Table 5: summary of the main findings in the repeated dose toxicity studies on rats

Annex 2 The Background Document (BD) gives the detailed scientific grounds for the opinion. The BD is based on the CLH report prepared by the Dossier Submitter; the evaluation performed by RAC is contained in 'RAC boxes'.

Annex 3 Comments received on the CLH report, response to comments provided by the Dossier Submitter and RAC (excluding confidential information).

ANNEX 1 – TABLE 5: SUMMARY OF THE MAIN FINDINGS IN THE REPEATED DOSE TOXICITY STUDIES ON RATS

Table 5: Summary for repeated dose toxicity studies in rats with isopyrazam. In all cases the difference with controls were statistically significant for at least $p<0.05$.

<table>
<tr><th>Method</th><th>Results</th><th>Reference</th></tr>
<tr><td>28-day oral (dietary)

OECD 407

Deviations: no FOB, only liver and uterus examined

GLP

HsdRccHan:WIST rats

5/sex/group

Isopyrazam (batch: SMU6AP001)

Purity: 96.4%

93:7 syn:anti
0, 300, 4000 and 8000 ppm

Males: 0, 29, 392 and 793 mg/kg bw/day

Females: 0, 28, 390 and 720 mg/kg bw/day</td><td>There were no deaths or clinical signs of toxicity

<u>793/720 mg/kg bw/day (M/F)</u>

Minimal/slight hepatocellular hypertrophy in all (10/10) animals

<table>
<tr><th></th><th>males</th><th>females</th></tr>
<tr><td>↓Terminal body weight</td><td>14%</td><td>18%</td></tr>
<tr><td>↑ Urea</td><td>-</td><td>34%</td></tr>
<tr><td>↑ Cholesterol</td><td>-</td><td>34%</td></tr>
<tr><td>↑ GGT</td><td>-</td><td>45%</td></tr>
<tr><td>↑ Phosphorous</td><td>-</td><td>44%</td></tr>
<tr><td>↓ Triglycerides</td><td>53%</td><td>-</td></tr>
<tr><td>↓ Creatinine</td><td>9%</td><td>-</td></tr>
<tr><td>↑ Relative liver weights</td><td>31%</td><td>28%</td></tr>
</table>

<u>392/390 mg/kg bw/day (M/F)</u>

Minimal/slight hepatocellular hypertrophy in all (10/10) animals

<table>
<tr><th></th><th>males</th><th>females</th></tr>
<tr><td>↓Terminal body weight</td><td>12%</td><td>10%</td></tr>
<tr><td>↑ Urea</td><td>-</td><td>20%</td></tr>
<tr><td>↑ Cholesterol</td><td>-</td><td>33%</td></tr>
<tr><td>↑ Phosphorous</td><td>-</td><td>24%</td></tr>
<tr><td>↓ Triglycerides</td><td>54%</td><td>-</td></tr>
<tr><td>↑ Relative liver weights</td><td>24%</td><td>22%</td></tr>
</table>

<u>29/28 mg/kg bw/day (M/F)</u>

No adverse effects</td><td>Anonymous (2007b)</td></tr>
</table>

28-day oral (dietary) OECD 407 Deviations: concentration, stability and homogeneity of test material not determined GLP HsdRccHan:WIST rats 5/sex/group Isopyrazam (batch: TE-5854/7) Purity: 100% **89:11 *syn*:*anti*** 0, 100, 500 and 2000 ppm Males: 0, 9, 46 and 175 mg/kg bw/day Females: 0, 10, 48 and 191 mg/kg bw/day	There were no deaths or clinical signs of toxicity <u>175/191 mg/kg bw/d (M/F)</u> Hepatocellular hypertrophy in all animals (10/10) |	males	females
↓ Terminal body weight	-	7%
↓ FC (week 4)	-	24%
↑ Creatinine	20%	-
↑ Creatinine kinase	56%	-
↓ Triglycerides	39%	-
↑ Relative liver weights	13%	14%
↑ P450 activity	200%	56% | <u>46/48 mg/kg bw/day (M/F)</u> ↑ 9% liver weights in females Hepatocellular hypertrophy in 1/5 males <u>9/10 mg/kg bw/day (M/F)</u> No adverse effects	Anonymous (2007a)
28-day oral (dietary) OECD 407 Deviations: No FOB GLP HsdRccHan:WIST RATS 5/sex/group (main study) and 6/sex/group (satellite phase) **Pure *Syn*** (SYN534969) (batch: SMU6BP001, (purity: 99%) **Pure *anti***	There were no deaths There was a dose-dependent increase in P450 activity for all test substances Hepatocellular hypertrophy was observed for all three test materials, in all treated males and at all dose levels and at the mid and high dose in females. The severity (minimal/slight/moderate) was related to dose level, except among the animals receiving 500 or 2000 ppm isopyrazam where the severity was similar. **Pure *syn*** <u>449.4/458.9 mg/kg bw/day (M/F)</u> |	males	females
↓ Platelets	12%	-
↓ WBC	34%	-
↓ Lymphocytes	36%	-
↓ Basophil	-	57%
↑ Cholesterol	-	40%
↓ ALP	31%	-
↑ Relative liver weight	25%	41% | <u>178.9/181.7 mg/kg bw/day (M/F)</u>	Anonymous (2007c)

(SYN534968) (batch: SMU6BP001, purity: 99.5%)

Isopyrazam (batch: SMU6CP014, purity: 98.2%)

***Syn*:*anti* ratio:50:50**

0, 500, 2000 and 5000 ppm

Males: 0, 47, 179 and 449 mg/kg bw/day

Females: 0, 47, 182 and 459 mg/kg bw/day

↑ Relative liver weight in males (17%) and females (31%)

47/46.8 mg/kg bw/day (M/F)

↑ Relative liver weight in females (22%)

Pure *anti*

406.5/372.3 mg/kg bw/day (M/F)

Hunched posture and piloerection in females

	males	females
↓ Terminal body weight	20%	18%
↑ Red blood cells	7%	-
↑ Haemoglobin	-	6%
↓ Basophil	-	61%
↓ Activated partial thromboplastin time	-	15%
↓ Total protein	-	6%
↓ Albumin	-	9%
↑ Cholesterol	-	54%
↑ Potassium	-	21%
↑ Phosphorous	-	36%
↓ Triglycerides	66%	-
↑ Relative liver weight	23%	38%

181.7/170 mg/kg bw/day (M/F)

Piloerection in females

	males	females
↓ Terminal body weight	11%	17%
↑ Red blood cells	-	8%
↓ Basophil	-	52%
↓ Activated partial thromboplastin time	-	13%
↓ Albumin	-	10%
↑ Cholesterol	-	40%
↓ Triglycerides	35%	-
↑ Relative liver weight	20%	20%

47/46.8 mg/kg bw/day (M/F)

	males	females
↓ Body weight (day 3)	-	3%
↓ Activated partial thromboplastin time	-	8%
↑ Relative liver weight	-	21%

50% *syn*:50% *anti*

456/371.9 mg/kg bw/day (M/F)

Hunched posture and piloerection (F)

	males	females
↓ Terminal body weight	14%	21%
↑ Red blood cells		9%
↓ Activated partial thromboplastin time	-	10%
↓ Total protein	-	6%
↓ Albumin	-	9%

↑ Cholesterol	-	63%
↑ Relative liver weight	30%	25%

181.1/197.8 mg/kg bw/day (M/F)

	males	females
↓ Terminal body weight	6%	11%
↑ Red blood cells	-	-
↓ Activated partial thromboplastin time	-	-
↓ Total protein	-	-
↓ Albumin	-	-
↑ Cholesterol	-	34%
↑ Relative liver weight	18%	34%

44.7/44.6 mg/kg bw/day (M/F)

	males	females
↓ Body weight (day 2)	-	4%
↑ Relative liver weight	-	15%

90-day oral (dietary)

OECD 408

GLP

HsdRccHan:WIST rats

12/sex/group

Isopyrazam (batch: SMU6AP001)

Purity: 96.4%

93:7 *syn*:*anti*
0, 300, 1500 and 6000 ppm

Males: 0, 21, 106 and 463 mg/kg bw/day

Females: 0, 24, 118 and 484 mg/kg bw/day

There were no deaths or clinical signs of toxicity

463/484 mg/kg bw/day

Minimal/slight hepatocellular hypertrophy in all animals

	males	females
↓ Body weight gain	19%	
↑ Cholesterol	-	27%
↑ Aspartate aminotransferase	17%	
↓ Gamma-glutamyl transferase	22%	26%
↓ Creatine kinase	47%	-
↑ Relative liver weight	24%	19%

106/118 mg/kg bw/day (M/F)

Minimal/slight hepatocellular hypertrophy in all animals

	males	females
↓ Body weight gain	-	7%
↑ Relative liver weight	14%	14%

21/24 mg/kg bw/day (M/F)

	males	females
↑ Relative liver weight	-	14%

Anonymous (2007d)

90-day oral (dietary)

OECD 408

GLP

Han Wistar (Crl:WI(Han) rats

10/sex/group

Isopyrazam (batch: SMU7DP017)

Purity: 96.4%

0, 100, 250 and 2000 ppm

93:7 *syn*:*anti*
Isopyrazam (batch: SMUDP017)

Purity: 90.8%

Males: 0, 8, 20 and 159 mg/kg bw/day

Females: 0, 8, 21 and 163 mg/kg bw/day

70:30 *syn*:*anti*

Males: 0, 10, 24 and 193 mg/kg bw/day

Females: 0, 9, 24 and 197 mg/kg bw/day

There were no deaths or clinical signs of toxicity

93:7 *syn*:*anti*

<u>159/163 mg/kg bw/day (M/F)</u>

	males	females
↓ Final body weight	-	18%
↑ Relative liver weight	18%	17%
↑ Thyroid weight	29%	-
Hepatocellular hypertrophy	6 mild + 2 moderate vs 0 controls	6 mild + 2 moderate vs 0 controls
Hepatocyte vacuolation	2 minimal + 1 mild versus 0 controls	2 minimal + 1 mild versus 0 controls

<u>20/21 mg/kg bw/day (M/F)</u>

No adverse effects

<u>8 mg/kg bw/day (M/F)</u>

No adverse effects

70:30*syn*:*anti*

<u>193/197 mg/kg bw/day (M/F)</u>

	males	females
↓ Final body weight	-	16%
↑ Relative liver weight	15%	15%
↑ Thyroid weight	29%	-
Hepatocellular hypertrophy	3 minimal + 5 mild vs 0 controls	3 minimal + 5 mild vs 0 controls
Hepatocyte vacuolation	2 minimal + 2 mild versus 0 controls	2 minimal + 2 mild versus 0 controls

<u>24/24 mg/kg bw/day (M/F)</u>

No adverse effects

<u>10/9 mg/kg bw/day (M/F)</u>

No adverse effects

Anonymous (2008)

90-day neurotoxicity (dietary)

OECD 424

GLP

HanRcc:WIST rats

12/sex/dose

Isopyrazam (batch: SMU6AP001)

Purity: 96.4%

There were no deaths or treatment-related clinical signs of toxicity

There were no FOB differences or ophthalmoscopy findings

There was no effect on brain weight and no treatment related macroscopic or microscopic effects in nervous system tissues

<u>382/468 mg/kg bw/day (M/F)</u>

↓ 28% Terminal body weight gain
↓ 14% Bodyweight gain (week 1)
Locomotor activity: ↓ distance traversed in females and ↓ number of rears in males and females at 13 weeks

<u>98/114 mg/kg bw/day (M/F)</u>

Anonymous (2009b)

93:7 *syn*:*anti* 0, 300, 1500 and 6000 ppm Males: 0, 20, 98 and 382 mg/kg bw/day Females: 0, 25, 114 and 468 mg/kg bw/day	No treatment-related effects <u>20/25 mg/kg bw/day (M/F)</u> No treatment-related effects	
2-year dietary toxicity and carcinogenicity study in Rats and histological extension OECD 453 GLP HsdRccHn:WIST rats 12/sex/dose (1-yr) 52/sex/dose (2-yr) 52/104 weeks exposure Isopyrazam (batch: SMU6AP001) Purity: 96.4% (w/w) **93:7 *syn*:*anti*** Doses: 0, 100, 500 and 3000 ppm Males: 0, 5.5, 28 and 173 mg/kg bw/day Females: 0, 7, 35 and 233 mg/kg bw/day	**Chronic phase (12 months)** There were no treatment-related deaths at any dose <u>173/233 mg/kg bw/day (M/F)</u>	Anonymous (2008a) and Anonymous (2009)

	males	females
↓ Mean body weight	10%	19%
↓ Hemoglobin	-	3%
↑ Platelet count	-	14%
↑ Gamma glutamyl transferase	13%	-
↓ Alkaline phosphatase	29%	42%
↓ Aspartate aminotransferase	-	35%
↑ Liver weight	17%	14%
Hepatocellular hypertrophy	5 minimal + 7 slight	12 slight
Hepatocyte pigmentation	5 minimal + 1 slight	10 minimal
Minimal hepatocyte vacuolation	11/12	8/12

<u>28/35 mg/kg bw/day (M/F)</u>

	males	females
↓ Mean body weight	-	4%
↑ Gamma glutamyl transferase	25%	
Hepatocellular hypertrophy	4 minimal	9 minimal
Hepatocyte pigmentation		
Minimal hepatocyte vacuolation	9 minimal + 1 slight	8 minimal

<u>5.5/7 mg/kg bw/day (M/F)</u>

No adverse effects

Carcinogenicity phase (24 months)

<u>173/233 mg/kg bw/day (M/F)</u>

	males	females
↓ Mean body weight	13%	27%
↓ Hemoglobin	-	5%
↓ Hematocrit	-	4%
↓ Red blood cells	-	6%
↑ Platelet count	-	11%

↑ Gamma glutamyl transferase	50%	43%
↓ Alkaline phosphatase	34%	31%
↓ Aspartate aminotransferase	-	21%
↑ Liver weight	18%	26%
Hepatocellular hypertrophy	49/52	50/52
Hepatocyte pigmentation	32/52	46/52
Minimal hepatocyte vacuolation	39/52	-
Minimal bile duct hyperplasia	12/52	-
Minimal bile duct fibrosis	12/52	-
Liver eosinophilic foci	32/52	29/52

28/35 mg/kg bw/day (M/F)

	males	females
↓ Mean body weight		11%
↑ Gamma glutamyl transferase	25%	-
↓ Alkaline phosphatase	17%	-
↓ Aspartate aminotransferase	-	22%
↑ Liver weight	5%	12%
Hepatocellular hypertrophy	45/52	49/52
Minimal hepatocyte vacuolation	32/52	8/52
Minimal bile duct hyperplasia	19/52	-
Minimal bile duct fibrosis	10/52	-
Liver eosinophilic foci	23/52	26/52

5.5/7 mg/kg bw/day (M/F)

	males	females
↓ Mean body weight		5%