Committee for Risk Assessment

RAC

Opinion

proposing harmonised classification and labelling
at EU level of

N-methoxy-*N*-[1-methyl-2-(2,4,6-trichlorophenyl)-ethyl]-3-(difluoromethyl)-1-methylpyrazole-4-carboxamide; pydiflumetofen

EC Number: -
CAS Number: 1228284-64-7

CLH-O-0000001412-86-271/F

Adopted

15 March 2019

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CLH-O-0000001412-86-271/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: **N-methoxy-N-[1-methyl-2-(2,4,6-trichlorophenyl)-ethyl]-3-(difluoromethyl)-1-methylpyrazole-4-carboxamide; pydiflumetofen**

EC Number: **-**

CAS Number: **1228284-64-7**

The proposal was submitted by **France** and received by RAC on **27 February 2018.**

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

PROCESS FOR ADOPTION OF THE OPINION

France 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 **9 April 2018**. Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by **8 June 2018**.

ADOPTION OF THE OPINION OF RAC

Rapporteur, appointed by RAC: **Brendan Murray**

Co-Rapporteur, appointed by RAC: **Anja Menard Srpčič**

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 **15 March 2019** by **consensus**.

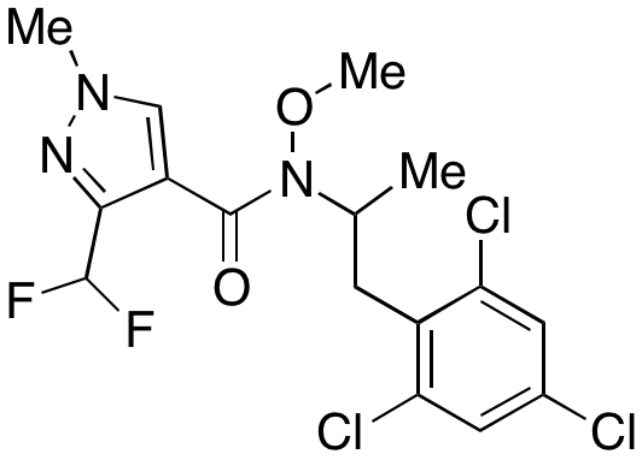
Classification and labelling in accordance with the CLP Regulation (Regulation (EC) 1272/2008)

	Index No	International Chemical Identification	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 submitter proposal	TBD	*N*-methoxy-*N*-[1-methyl-2-(2,4,6-trichlorophenyl)-ethyl]-3-(difluoromethyl)-1-methylpyrazole-4-carboxamide; pydiflumetofen	-	1228284-64-7	Aquatic Acute 1 Aquatic Chronic 1	H400 H410	GHS09 Wng	H410		M=1 M=1	
RAC opinion	TBD	*N*-methoxy-*N*-[1-methyl-2-(2,4,6-trichlorophenyl)-ethyl]-3-(difluoromethyl)-1-methylpyrazole-4-carboxamide; pydiflumetofen	-	1228284-64-7	Carc. 2 Repr. 2 Aquatic Acute 1 Aquatic Chronic 1	H351 H361f H400 H410	GHS08 GHS09 Wng	H351 H361f H410		M=1 M=1	
Resulting Annex VI entry if agreed by COM	TBD	*N*-methoxy-*N*-[1-methyl-2-(2,4,6-trichlorophenyl)-ethyl]-3-(difluoromethyl)-1-methylpyrazole-4-carboxamide; pydiflumetofen	-	1228284-64-7	Carc. 2 Repr. 2 Aquatic Acute 1 Aquatic Chronic 1	H351 H361f H400 H410	GHS08 GHS09 Wng	H351 H361f H410		M=1 M=1	

GROUNDS FOR ADOPTION OF THE OPINION

RAC general comment

Pydiflumetofen is a new pesticidal active substance in the scope of Regulation 1107/2009. It is a broad-spectrum fungicide for use on various field crops to control for powdery mildew (*Uncinula necator*); Septoria (*Septoria tritici*); Target spot/early blight (*Alternaria solani*); Scab (*Venturia pyrina*) & Grey mould (*Botrytis cinerea*). The active substance is composed of two enantiomers (the S-isomer and R-isomer, present in a 1:1 ratio) marketed as a racemate mixture. It is a pyrazole-carboxamide fungicide that stunts fungus growth by inhibiting succinate dehydrogenase, complex II in the mitochondrial respiration chain, which in turn interferes with the tricarboxylic cycle and mitochondrial electron transport (it therefore belongs to the class of succinate dehydrogenase inhibitors or SDHI fungicides). It interferes with several key fungal life functions, including mycelial growth and conidium germination. It has no current entry in Annex VI of the CLP regulation and all hazard classes are open for assessment in this opinion document.



The toxicological database for pydiflumetofen consists of toxicity studies currently required for hazard assessment purposes, as well as several mechanistic studies to support a proposed mode of action (MOA) for liver tumour formation in mice. The studies were carried out in accordance with currently accepted international testing protocols and Good Laboratory Practice. The available information is considered adequate for characterising the potential health hazards associated with this active ingredient.

The DS presented the CLH report according to the new combined DAR/CLH report template to align the process of substance evaluation under Regulation 1107/2009 through EFSA while simultaneously producing an integrated CLH report to satisfy the process for CLP.

RAC evaluation of physical hazards

Summary of the Dossier Submitter's proposal

The Dossier Submitter (DS) does not propose classification of pydiflumetofen for physical hazards on the basis of the following results:

- Negative results in a UN Test.2 (b) & (c) study (*Jackson, 2016*) for testing the capability of pydiflumetofen to be explosive;
- Negative results in a measured ASTM E537/ UN Test N.1 study (*Jackson, 2016*) for testing the flammability of pydiflumetofen;
- Measured EC A.16 test, no self-ignition was detected;
- Measured UN Test O.1 (*Jackson, 2016*) indicated that pydiflumetofen is not oxidising.

Pydiflumetofen is an opaque solid in the form of a fine, non-free flowing powder. Pydiflumetofen melted at 112.7°C. No decomposition occurred below the melting point. The DS considered pydiflumetofen was not flammable, not auto-flammable, nor explosive and had no oxidising properties.

Comments received during public consultation

No comments received.

Assessment and comparison with the classification criteria

Criteria for classification of physical hazards have not been satisfied based on the data obtained from several key studies. RAC agrees with the DS proposal for **no classification for physical hazards**.

HUMAN HEALTH HAZARD EVALUATION

RAC evaluation of acute toxicity

Summary of the Dossier Submitter's proposal

The DS proposed no classification of pydiflumetofen with acute oral toxicity based on one negative study performed with Wistar female rats according to GLP and OECD TG 425 (*Anon., 2012*). LD_{50} > 5000 mg/kg bw. Two acute neurotoxicity studies performed according to GLP and OECD TG 424 (*Anon. 2015a, b*) were also assessed: a single female out of 10 dosed at 1000 mg/kg bw was killed due to the severity of clinical signs (no deaths amongst males, *Anon. 2015a*), with no deaths at the high dose of 2000 mg/kg bw. In the second acute neurotoxicity study (*Anon. 2015b*) there were no deaths up to the maximum tested dose of 1000 mg/kg/day. These data are consistent with an LD_{50} > 2000 mg/kg bw and > 1000 mg/kg/day respectively.

The DS proposed no classification of pydiflumetofen for acute dermal toxicity on the basis of no lethalities at the limit dose (5000 mg/kg bw) in a GLP and OECD guideline 402 study (*Anon. 2013*; semi occlusive, 24 hour exposure to 5 male and 5 female CRL:(WI) rats, followed by a 14-day observation period).

The DS proposed no classification for acute inhalation toxicity. In an OECD 403 acute inhalation study (*Anon, 2013*), groups of 5 CRL: (WI) Wistar strain rats/sex/dose were nose-only exposed for 4 h to a dust aerosol of pydiflumetofen at a concentration of 5.11 ± 0.18 mg/L (gravimetrically determined). One female was found dead after exposure. Diffuse, dark red discoloration of the non collapsed lungs were seen in this rat at necropsy, the relationship of these findings to treatment was uncertain. The particle size of the test atmosphere was 3.54 µm MMAD.

Comments received during public consultation

No comments received.

Assessment and comparison with the classification criteria

(1) Acute oral toxicity: The oral LD_{50} of > 2000 mg/kg bw for rats is above the value for classification according to CLP guidance.

(2) Acute dermal toxicity: The dermal LD_{50} of > 5000 mg/kg bw for rats is above the value for classification according to CLP guidance.

(3) Acute inhalation toxicity: The 4 h inhalation LC_{50} of > 5.11 mg/L for rats is above the value for classification in the CLP Regulation (i.e. 5 mg/L dust).

RAC considers that the substance **does not warrant classification for acute toxicity** via the oral, dermal and inhalation routes.

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

Summary of the Dossier Submitter's proposal

1. Relevance of the provided information on STOT-SE

Acute Neurotoxicity in the rat

The DS described two acute neurotox studies (*Anon., 2015a, 2015b*) followed by an assessment of the evidence from supporting repeat dose studies such as the FOB (functional observation battery) information in the 90-day and 2-year toxicity studies in rats.

In the acute studies, single gavage doses of 0, 100, 300, 1000 and 2000 mg/kg produced some clinical signs at dose levels ≥ 300 mg/kg in females only. Clinical signs of neurotoxicity were seen within 2-6 hours of dosing at 300 mg/kg and above (recumbency, piloerection, reduced activity, abnormal gait, skin cold to touch; pupillary reflex impaired and mydriasis, reduced body temperature; decrease in locomotor activity). These effects were transient and rapidly reversible. No gross or histopathological findings in the central or peripheral nervous system were observed.

Other evidence

The DS found no evidence within the FOB information in the 90-day and 2-year toxicity studies in rats to support effects on locomotor activity that might warrant classification for STOT SE.

In the acute oral toxicity study, 3 female CRL:(WI) rats were given a single oral (gavage) dose of 5000mg/kg/day. Treatment caused a slight decrease in activity in one animal.

In the acute inhalation toxicity study, 10 (5 male and 5 female) CRL: (WI) Wistar strain rats, were exposed to an aerosol concentration of 5.11 mg/L pydiflumetofen. The animals were

exposed for 4 hours using a nose-only exposure system. Laboured, gasping and noisy respiration, sneezing, decreased activity, prostration and ataxia were recorded for the exposed animals on the day of exposure. One female was found dead after exposure. Noisy respiration or weakness were recorded in 3 males and 3 females the day after exposure, weakness was noted in 2 males and 1 female 2 days after exposure and no significant clinical signs were observed from Day 3 until scheduled necropsy.

Conclusion of the Dossier Submitter

Overall, the DS concluded that the results from the standard acute and acute neurotoxicity studies did not indicate that there was specific organ toxicity following a single exposure. In addition, very transient and slight clinical signs reported for narcotic/neurotoxic effects did not fulfil the criteria for STOT SE.

Comments received during public consultation

No comments were received.

Assessment and comparison with the classification criteria

Introduction

According to CLP, STOT-SE should be considered where there is a clear evidence for specific organ toxicity, especially when it is observed in absence of lethality. It is defined as specific, non-lethal target organ toxicity arising from a single exposure to a substance or mixture. All significant health effects that can impair function, reversible and irreversible, immediate and/or delayed are considered.

STOT SE 1 or 2

A decrease in female locomotor activity (LMA, distance travelled and number of rears), at 6 hrs post-dose on day 1 was observed in one of the acute neurotoxicity studies at a dose (300 mg/kg bw) which was within the guidance value range for STOT SE 1 (C ≤ 300 mg/kg bw). This effect was observed without any further impact on health and was only observed on the day of dosing. The difference in LMA relative to the control group did not attain statistical significance. No gross or histopathological findings in the central or peripheral nervous system were seen. Such effects were not observed in the 90-day toxicity study in female rats at doses up to 1174 mg/kg/day. RAC does not consider this effect sufficient for classification as STOT-SE 1 or 2.

STOT SE 3

The hazard class STOT SE 3 should cover 'transient' narcotic effects occurring after single exposure. Such narcotic effects observed in animal studies may include lethargy, lack of coordination, loss of righting reflex, and ataxia. In the oral single dose studies, some symptoms were observed in rats (decreased locomotor activity). These clinical signs occurred rapidly after dosing, appeared to be unspecific and were transient in nature. RAC is of the opinion that the very transient and slight reported LMA signs **do not fulfil the criteria for STOT SE 3 and does not propose classification**.

RAC evaluation of skin corrosion/irritation

Summary of the Dossier Submitter's proposal

The DS described a primary dermal irritation study (GLP, OECD TG 404, *Anon, 2012*) where young adult New Zealand white rabbits (3 males) were exposed to 0.5 g pydiflumetofen, applied to the intact shaved flank under a semi-occlusive dressing, for 4 hours. Skin reactions were scored at 1, 24, 48 and 72 hours after removal of the dressings. No clinical signs were observed in the animals during the study and no mortality occurred. No local dermal clinical signs were observed in the treated animals throughout the study. The primary irritation index (calculated by totalling the mean cumulative scores at 24, 48 and 72 hours for all animals and then dividing by the number of data points) was 0.00 and no corrosive effects were noted on the treated skin of any animal at any of the observation intervals.

Mean scores / animal (24, 48 and 72 hours)

Erythema: 0, 0, 0;

Oedema: 0, 0, 0

Comments received during public consultation

No comments were received.

Assessment and comparison with the classification criteria

There was no evidence of a skin reaction in any of the treated animals (mean scores for erythema and oedema 0), therefore the data **do not meet the criteria for classification and labelling for skin corrosion/irritation**.

RAC evaluation of serious eye damage/irritation

Summary of the Dossier Submitter's proposal

In a GLP, OECD TG 405 (2002) compliant primary eye irritation study (*Anon., 2012b*), minor, transient signs of ocular irritation were observed. At 1 hour after treatment, discharge was observed in one rabbit (score 1) and conjunctival redness was seen in all rabbits (two with a score 2 and one rabbit had a score 1).

Conjunctival redness (score 1) was seen in one rabbit at 24 and 48 hours after treatment. All symptoms had fully reversed in all animals at the 72 hour observation. No clinical signs of systemic toxicity were observed in the animals during the study.

Mean scores for corneal opacity, iritis and chemosis were 0 in all animals. The mean score for conjunctival redness (after 24 to 72 hours) was 0 in two rabbits and 0.67 in one rabbit.

The DS did not propose classification.

Comments received during public consultation

No comments were received.

Assessment and comparison with the classification criteria

The mean scores in all animals were negative (corneal opacity < 1; iritis < 1; conjunctival redness < 2; chemosis < 2). The data **do not meet the criteria for classification of serious eye damage/irritation**.

RAC evaluation of respiratory sensitisation

Summary of the Dossier Submitter's proposal

There were no specific studies performed with pydiflumetofen. The DS commented that there was no evidence from single or repeated dose animal studies or from occupational monitoring (during active ingredient manufacture, subsequent formulation and field trials) that pydiflumetofen had any potential to cause respiratory sensitisation.

Comments received during public consultation

No comments received.

Assessment and comparison with the classification criteria

There is no evidence or data on respiratory sensitisation. **RAC concludes on no classification due to lack of data.**

RAC evaluation of skin sensitisation

Summary of the Dossier Submitter's proposal

Skin sensitisation potential was assessed in a GLP and OECD TG 429 (2010) compliant, mouse Local Lymph Node Assay (*Anon., 2013*) using groups of five female CBA/J Rj mice. The criterion for a positive response is one or more of the concentrations tested should elicit a 3-fold or greater increase in isotope incorporation relative to the vehicle control group. The test substance was applied as 50, 25 and 10 % (w/v) pydiflumetofen preparations in acetone:olive oil 4:1 (v:v). A positive control group received 25% α-Hexylcinnamaldehyde (HCA) in the same vehicle mixture.

No mortality or signs of systemic toxicity were observed during the study. There were no indications of any irritancy at the site of application. Stimulation index values of the test item were 1.0, 1.1 and 1.1 at concentrations of 50, 25 and 10% (w/v) respectively, indicating that pydiflumetofen was shown to be a non-sensitiser in the Local Lymph Node Assay. In the positive control group, α-Hexylcinnamaldehyde induced a positive response with a stimulation index of 7.4, confirming the validity of the protocol used in this study.

The DS did not propose classification for skin sensitisation.

Comments received during public consultation

No comments received.

Assessment and comparison with the classification criteria

As no evidence of skin sensitisation was observed in the Local Lymph Node Assay in the mouse (i.e. the data gave a stimulation index < 3), the criteria for classification according to CLP were not met. RAC concludes that **no classification for skin sensitization is warranted**.

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

Summary of the Dossier Submitter's proposal

The DS did not propose classification for STOT RE. The repeated dose toxicity of pydiflumetofen was investigated in several species (rats, mice and dogs). The liver was identified as the target organ with a consistent pattern of increased liver weight associated with histopathological changes and/or modified clinical chemistry. In addition, lower body weight gains were observed in all species and thyroid hypertrophy was only observed in rats. All these effects were observed at doses above the critical guidance values for STOT RE 2. The DS concluded that repeated dosing with pydiflumetofen did not produce results that indicated severe or toxicologically relevant effects on organs at dose levels below the guidance values for classification.

Table 31 in the CLH report summarises the repeat dose studies on pydiflumetofen which were conducted in rats (28-day dietary, 28-day dermal, 90-day dietary, 2-year dietary combined chronic toxicity/ carcinogenicity study, 2-generation reproductive dietary study), mice (28-day dietary, 90-day dietary) and dogs (90-day oral (capsule), 1-year oral (capsule)).

Comments received during public consultation

No comments were received.

Assessment and comparison with the classification criteria

Comparison with the criteria

***Table**: Summary of the most relevant effects for consideration of STOT-RE in doses requiring comparison to the trigger dose values.*

Study	Relevant effect level	Cat. 1 mg/kg/day	Cat. 2 mg/kg/day	Significant & Potentially Relevant Effects (dose response? Y/N)	Reference
Rat, 28 day oral dietary **Not sufficient for classification**	4000 ppm **Males**: 343 mg/kg/day **Females**: 322 mg/kg/day	≤ 30	≤ 300	**Males**: (control vs 4000 ppm) ↑ Abs. liver wt. (+19%)** (Y) ↑ Rel. liver wt. (+21%) (Y) Liver: ↑ hypertrophy: 0 vs 4 (min)[1] (Y) **Females**: (control vs 4000 ppm) ↑ Abs. liver wt. (+20%)* (Y) ↑ Rel. liver wt. (+24%) (Y)	DAR B.6.3.1 (K-CA 5.3.1/01)

Rat, 90 day oral dietary **Not sufficient for classification**	1500 ppm **Males**: 111 mg/kg/day **Females**: 127 mg/kg/day	≤ 10	≤ 100	**Males**: (control vs 1500 ppm) ↑ Abs. liver wt. (+21%)* (Y) ↑ Rel. liver wt. (+26%) (Y) Liver: ↑ hypertrophy: 0 vs 5* (min)[2] (Y) Thyroid: ↑ follicular hypertrophy: 0 vs 4 (min)[2] ↓ serum ALP (-30%)** **Females**: (control vs 1500 ppm) ↑ Abs. liver wt. (+16%)** (Y) ↑ Rel. liver wt. (+18%) (Y) ↓ serum ALP (-41%)**	DAR B.6.3.2.1 (K-CA 5.3.2/01/02)
Mouse, 28 day oral dietary **Not sufficient for classification**	1500 ppm **Males**: 213 mg/kg/day **Females**: 266 mg/kg/day	≤ 30	≤ 300	**Males**: (control vs 1500 ppm) ↓ bw gain (-16%) (N) ↑ Abs. liver wt. (+25%)** (Y) ↑ Rel. liver wt. (+31%)** (Y) No histopathological findings. **Females**: (control vs 1500 ppm) ↓ bw gain (-23%) (N) ↑ Abs. liver wt. (+23%) (Y) ↑ Rel. liver wt. (+30%) (Y) No histopathological findings.	DAR B.6.3.1 (K-CA 5.3.1/02)
Mouse, 90 day oral dietary **Not sufficient for classification**	500 ppm **Males**: 81.6 mg/kg/day	≤ 10	≤ 100	**Males**: (control vs 500 ppm) ↑ Abs. liver wt. (+18%)* (Y) ↑ Rel. liver wt. (+14%) (Y) Liver: ↑ hypertrophy: 0 vs 2/10 (mild) (Y) No other histopathological findings	DAR B.6.3.2.2 (K-CA 5.3.2/03/04)
Dog, 90 day oral (capsule) **Not sufficient for classification**	30 mg/kg/day	≤ 10	≤ 100	**Males**: No effects. **Females**: No effects.	DAR B.6.3.2.3 (IIA 5.3.2/05)
Dog, 12 month oral dietary **Not sufficient for classification**	30 mg/kg/day	≤ 2.5	≤ 25	**Males**: (control vs 30 mg/kg/day) ↑ Rel. liver wt. (+19%) (Y) ↑ Rel. thyroid wt. (+29%) (Y) **Females**: (control vs 30 mg/kg/day) ↑ Rel. liver wt. (+22%) (Y)	DAR B.6.3.2.3 (IIA 5.3.2/06)
Rat, 28 day dermal **Not sufficient for classification**	300 mg/kg/day	≤ 60	≤ 600	**Males**: No effects. **Females**: No effects.	DAR B.6.3.3 (IIA 5.3.3/01)

2 year dietary study in rats **Not sufficient for classification**	200 ppm **Males**: 9.9 mg/kg/day	≤ 1.25	≤ 12.5	**Males**: (control vs 200 ppm) Liver: ↑ prominent lobular architecture: 5 vs 15 (animal incidence) (N)	DAR B.6.5.1 (IIA 5.5/01)
18 month dietary study in mice **Not sufficient for classification**	75 ppm (lowest dose tested) **Males**: 9.2 mg/kg/day **Females**: 9.7 mg/kg/day	≤ 1.67	≤ 16.7	**Males**: No effects. **Females**: No effects.	DAR B.6.5.2 (IIA 5.5/03)
Two generation reproduction Rat, oral dietary **Not sufficient for classification**	750 (M)/ 450 (F) ppm **Males**: F0: 46 mg/kg/day F1: 59 mg/kg/day **Females**: F0: 36 mg/kg/day F1: 42 mg/kg/day	≤ 10	≤ 100	**Males**: (control vs 750 ppm) F0/ F1: No adverse effects. **Females**: (control vs 450 ppm) F0/ F1: No adverse effects.	DAR B.6.6.1 (IIA 5.6.1/01)

* significantly different from control, p ≤ 0.05

** significantly different from control, p ≤ 0.01

[1]animals affected out of 6 (min = minimal grading)

[2]animals affected out of 10 (min = minimal grading)

The table above presents the most pertinent data for consideration of STOT-RE classification. Based on the data neither a category 1 or a category 2 classification is warranted. Severe toxicological effects were not demonstrated at or below the guidance critical values. The target organ in all species is the liver. Table 31 in the CLH report provides a greater level of detail for all effects at all dose levels in those studies most suitable for STOT RE.

RAC concludes that repeated dosing with pydiflumetofen produced no effects that were indicative of organ dysfunction at dose levels below the guidance value for classification as STOT-RE, therefore **no classification is proposed**.

RAC evaluation of germ cell mutagenicity

Summary of the Dossier Submitter's proposal

The DS reported that pydiflumetofen was tested in a range of *in vitro* and *in vivo* genotoxicity assays (see also the table below).

In vitro assays included:

- two *in vitro* Ames tests (reverse mutation assay with *Salmonella typhimurium* and *Escherichia coli*) (*Sokolowski*, 2012, 2014),

- an *in vitro* cell gene mutation test (in mouse lymphoma L5178Y cells) (*Wollny*, 2013),
- an *in vitro* chromosome aberration test (in human lymphocytes) (*Bohnenberger*, 2013).

In vivo assays included:

- two *in vivo* micronucleus assays in mouse rat bone marrow (*Anon.*, 2012, 2014),
- an *in vivo* rat bone marrow chromosome aberration test (not reported in the CLH report, but it is reported in the 2018 DAR) (*Anon.*, 2017),

***In vitro* results**

Pydiflumetofen was found to be negative in two reverse mutagenicity tests in bacteria with and without metabolic activation. In a mutagenicity study in mammalian cells, testing for forward mutation in mouse lymphoma cells, pydiflumetofen did not increase the mean mutation frequency in the presence or absence of S9-mix. However, the clastogenic effect of pydiflumetofen was tested in an *in vitro* chromosome aberration study in human lymphocytes (*Bohnenberger, 2013*). In 2 of 3 experiments, in the absence of S9 mix, there were statistically significant increases in chromosomal aberrations.

***In vivo* results**

The clastogenic effect of pydiflumetofen was further investigated *in vivo*. In the *in vitro* test in human lymphocytes there was some evidence of chromosome damage in cultures without metabolic activation (this was considered negative by most of the experts during a recent EFSA expert meeting (PPR 182, September 2018)). *In vivo* pydiflumetofen did not induce micronuclei in the polychromatic erythrocytes of the bone marrow of mice. The mean number of polychromatic erythrocytes was not decreased after treatment indicating that pydiflumetofen did not have any significant cytotoxic properties in the bone marrow.

Conclusion

According to the DS, when considering all the data, pydiflumetofen did not present a genotoxic hazard. There were no studies in germ cells. The DS did not propose to classify pydiflumetofen as mutagenic.

***Table**: Summary of genotoxicity tests with pydiflumetofen adapted from table 35 and 36 in the CLH report, and table 6.4-1 in the DAR.*

Study	Result	Test System	Reference
***In vitro* studies:**			
Bacterial mutagenicity	negative	GLP, OECD TG 471 (1997) *Salmonella* Strains: TA1535, TA1537, TA98, TA100 *E. coli* strains WP2uvrApKM101 and WP2pKM101	*Sokolowski,* (2012)
Bacterial mutagenicity	negative	GLP, OECD TG 471 (1997) *Salmonella* Strains: TA1535, TA1537, TA98, TA100 *E. coli* strains WP2uvrApKM101 and WP2pKM101	*Sokolowski,* (2014)

Mammalian cell mutagenicity	negative	GLP, OECD TG 476 (1997) Mouse Lymphoma L5178Y Cells (Thymidine Kinase locus)	*Wollny,* (2013)
Clastogenicity	Positive or equivocal?	GLP, OECD TG 473 (1997) cultured human lymphocytes	*Bohnenberger,* (2013)
***In vivo* studies:**			
Micronucleus	negative	GLP, OECD TG 474 (1997) Male NMRI mouse bone marrow (short term)	*Anonymous*, (2012)
Micronucleus	negative	GLP, OECD TG 474 (1997) Male NMRI mouse bone marrow (short term)	*Anonymous*, (2014)
Clastogenicity (chromosome aberration assay)	negative	GLP, OECD TG 475 (2016) Han Wistar rat, male (single oral gavage); bone marrow	*Anonymous,* (2017)

Comments received during public consultation

One MSCA supported the proposal not to classify pydiflumetofen for mutagenic effects. One other comment was made by the same MSCA in relation to a proposed request for an additional study – a comet assay. They did not support the request for a new study.

One Company-Manufacturer provided an additional *in-vivo* rat bone marrow chromosome aberration assay on the batch of material (SMU2EP12007) that gave a positive response in the *in-vitro* chromosome aberration test in human lymphocytes.

Assessment and comparison with the classification criteria

In Vitro Tests

Summary

Pydiflumetofen gave negative responses in the Ames tests and the mouse lymphoma L5178Y test, and a positive result in the *in vitro* chromosome aberration test in human lymphocytes; indicative of an *in vitro* clastogenic effect.

Bacterial and mammalian cell mutagenicity tests

The Ames studies were conducted on the toxicology batch of material (SMU2EP12007; *Sokolowski, 2012*) and on a batch of material spiked with potential impurities (SMU4FL762; *Sokolowski, 2014*) to support the technical specification of pydiflumetofen. No substantial increase in revertant colony numbers of any of the six tester strains was observed following treatment at any dose level (up to 5000 µg/plate), either in the presence or absence of metabolic activation (S9 mix). Appropriate reference mutagens were used as positive controls and showed an increase in induced revertant colonies.

Pydiflumetofen did not induce mutations in the mouse lymphoma thymidine kinase locus assay using the cell line L5178Y in the absence and presence of metabolic activation (*Wollny, 2013*). Methyl methanesulphonate (19.5 µg/mL) and cyclophosphamide (3.0 and 4.5 µg/mL) were used as positive controls and showed an increase in induced total mutant colonies within acceptable levels of toxicity.

RAC agrees with the DS, pydiflumetofen shows no potential for *in vitro* mutagenicity.

Mammalian cell chromosome aberration test

A GLP, guideline compliant *in vitro* chromosome aberration test in human lymphocytes (*Bohnenberger, 2013*) in the absence of S9 mix, showed a weak positive response at a low concentration of 5.3 µg/mL in a series of tested concentrations after 22 hours of exposure. Tests at 9.2 and 16.1 µg/mL were negative.

Layout of the experiments in the *Bohnenberger* (2013) study (positive aberrations associated with concentrations in bold):

- Experiment I:
  - exposure 4 hr: [16.1 - 28.1 - 150.5] µg/mL; without S9 mix
  - exposure 4 hr: [16.1 – 28.1 – 49.2] µg/mL; with S9 mix
- Experiment IIA:
  - exposure 22 hr: [**5.3** – 9.2 – 16.1] µg/mL; without S9 mix
  - exposure 22 hr: [9.2 – 16.1 – 2475 - 4332] µg/mL; with S9 mix
- Experiment IIB: (confirmatory experiment)
  - exposure 22 hr: [3.0 – 4.0 – 5.0 – 6.0 – 7.0 – 10.0 – 15.0 – **20.0** – **40.0**] µg/mL; without S9 mix

Experiment I is a short term (4 hr exposure ± S9 mix) test. Experiment IIA is a 22 hr exposure test (-S9 mix) and a 4 hr exposure test (+S9 mix). Experiment IIB is a 22 hr confirmatory test (-S9 mix) to investigate if the chromosomal aberrations observed with pydiflumetofen in the absence of S9 mix were repeatable.

In Experiment IIA in the absence of S9 mix, one statistically significant increase (6.5 % aberrant cells, excluding gaps) above the range of the laboratory historical solvent control data (0.0 - 3.0% aberrant cells, excluding gaps), was observed after treatment with 5.3 µg/mL (table below). No dose-dependency was observed. Based on this weak positive result, a confirmatory test (Experiment IIB) was conducted. Statistically significant increases occurred after treatment with 20.0 and 40.0 µg/mL (7.5 and 9.5 % aberrant cells, excluding gaps respectively) and the results clearly exceeded the laboratory historical solvent control range of 0.0 - 3.0% (table below).

There was no evidence of an increase in polyploid metaphases after treatment with the test substance relative to the control cultures. Positive controls (ethyl methanesulphonate [-S9 clastogen] and cyclophosphamide [+S9 clastogen]) behaved appropriately and showed clear increases in cells with structural chromosome aberrations. Cytotoxicity was not an issue at the concentrations of test substance employed in the study. Precipitates were visible at the highest concentrations tested.

Conclusion

The *in vitro* mutagenicity tests were all negative. Pydiflumetofen induced structural chromosomal aberrations in human lymphocytes *in vitro* in the absence of metabolic activation. The responses were not linear but were dose related and clearly outside the distribution of the historical negative control data. The study was acceptable from a regulatory point of view and is a recent GLP and guideline compliant investigation. RAC considers the data weakly positive to equivocal regarding evidence for chromosomal aberration and places more weight on the *in vivo* studies for the overall assessment for the potential for clastogenicity. The potential for chromosomal aberrations was further investigated in two *in vivo* mouse micronucleus studies and one *in vivo* rat bone marrow chromosome aberration assay. These are discussed below.

Table: Summary of results of the in vitro chromosomal aberration study with pydiflumetofen

Exp.	Preparation interval	Test substance concentration in µg/mL	Mitotic indices in % of control	Aberrant cells in % incl. gaps*	Aberrant cells in % excl. gaps*	Aberrant cells in % carrying exchanges
Exposure period 4 hrs without S9 mix						
I	22 hrs	Solvent control[1]	100.0	0.5	0.5	0.0
		Positive control[2]	86.5	10.0	**9.5[S]**	1.0
		16.1	84.5	1.0	1.0	0.0
		28.1[P]	88.1	2.0	2.0	0.0
		150.8[P]	94.4	1.0	1.0	0.0
Exposure period 22 hrs without S9 mix						
IIA	22 hrs	Solvent control[1]	100.0	0.5	0.5	0.0
		Positive control[2#]	68.9	43.0	**42.0[S]**	17.0
		5.3[##]	89.4	6.5	**6.5[S]**	0.0
		9.2	104.4	2.0	1.5	0.0
		16.1[P]	105.6	1.0	1.0	0.0
IIB	22 hrs	Solvent control[1]	100.0	1.5	1.0	0.0
		Positive control[2]	37.6	21.5	**21.5[S]**	6.5
		3.0	117.4	0.5	0.5	0.0
		4.0	110.3	2.0	2.0	0.0
		5.0	111.6	1.0	1.0	0.0
		6.0	106.6	0.5	0.5	0.0
		7.0	83.5	0.5	0.5	0.0
		10.0	95.5	0.0	0.0	0.0
		15.0[##]	76.9	3.5	3.0	0.3
		20.0[P##]	76.0	7.8	**7.5[S]**	0.3
		40.0[P]	73.1	10.5	**9.5[S]**	0.0

[1]DMSO 1.0% (v/v); [2]EMS (770 µg/ml); [P]Precipitation occurred at the end of treatment; [S]Statistically significant

***In Vivo* Tests**

Summary

As determined by a pre-experiment in male and female mice, 2000 mg pydiflumetofen per kg bw was considered suitable by the applicant as the highest dose. Since no obvious gender-specific differences in the sensitivity to the test substance were observed, the Sponsor performed the main experiment using male animals only. Pydiflumetofen did not induce micronuclei in the polychromatic erythrocytes of the bone marrow of mice.

In vivo mouse bone marrow micronucleus tests

Two *in vivo* mouse bone marrow micronucleus tests were conducted, one of which was on the batch of pydiflumetofen (SMU2EP12007) that gave a positive response in the *in vitro*

chromosome aberration test. The second study was conducted on a batch of material spiked with potential impurities (SMU4FL762) to support the technical specification of the test substance. Both *in vivo* micronucleus tests gave negative results. These studies demonstrated that the positive *in vitro* clastogenicity finding for pydiflumetofen was not corroborated *in vivo*. The *in vivo* mouse bone marrow micronucleus tests did not include an assessment of bone marrow exposure to pydiflumetofen, however in both *in vivo* studies the observed clinical signs at the highest dose were typically indicative of systemic absorption of the test material. No dose dependence was observed in either study. A dose of 40 mg/kg bw cyclophosphamide administered orally was used as the positive control, which showed a statistically significant increase of induced micronuclei.

The plant protection product DAR summarised extensive toxicokinetic investigations into pydiflumetofen in both rats and mice (DAR section B.6.1.1). Following gavage dosing pydiflumetofen and its metabolites were shown to be present in blood and plasma over at least a 24 hour period post-dosing in both species. The bone marrow may be considered to have been sufficiently exposed to pydiflumetofen and its metabolites to reliably assess clastogenicity and aneugenicity. Evaluation of the pharmacokinetics of pydiflumetofen in the mouse following one of the dose regimes with 1000 mg/kg bw (DAR B.6.1.1, *Anon, 2014a*; K-CA 5.1.1/07) revealed pydiflumetofen was quantifiable in blood at all sampling timepoints (0.5, 1, 2, 4, 8, 12 and 24 hours). The increase in systemic exposure was non-linear with respect to dose in male mice beyond *ca.* 100 mg/kg. As all doses in the micronucleus assays were above 100 mg/kg, exposure of the bone marrow to pydiflumetofen was assumed.

In vivo rat bone marrow chromosome aberration assay

During the peer review of pydiflumetofen within the scope of Regulation 1107/2009 for the evaluation of plant protection products, the industry applicant supplied a third *in vivo* study to the Rapporteur Member State (RMS) for inclusion into the DAR (*Anon, 2017*). This study, a rat bone marrow chromosome aberration assay, was performed according to GLP and OECD TG 475 (2016) compliant. Exposure consisted of a single dose administered by oral gavage.

The limit dose of 2000 mg/kg was well tolerated in males and females and was selected as the maximum dose level for the main experiment. Intermediate dose levels of 1000 and 500 mg/kg were also selected. In the absence of a difference in tolerability between males and females, the main study was conducted in male Crl:WI(Han) Wistar rats only.

Bone marrow was sampled at two time points: 16 hours and 42 hours after test substance administration. At both time-points the mean percentage of cells with aberrations (excluding gaps) was not substantially changed from controls (table below).

16 hour data

For dose levels of 500, 1000 and 2000 mg/kg, cytotoxicity (as measured by mitotic inhibition [%MIH]), was 6%, 24% and 27% respectively, providing weak evidence of bone marrow toxicity at 2000 mg/kg. At the 16 hour sample time, the mean percentage of cells with aberrations (excluding gaps) was 0.42%, 0.42% and 0.72% at 500, 1000 and 2000 mg/kg, respectively, compared to 0.42% in the concurrent vehicle control (not statistically significant). For dose groups of 500, 1000 and 2000 mg/kg, mean numerical aberration frequencies were 0.9%, 0.6% and 0.6% respectively compared to 0.4% in the concurrent vehicle control. Overall, frequencies of cells with numerical aberrations in all treated groups fell within the expected historical control range.

42 hour data

At 42 hours, cytotoxicity (as measured by %MIH), at 2000 mg/kg was 17%. At the 42 hour sample time, the mean percentage of cells with aberrations (excluding gaps) was 0.58% at 2000 mg/kg compared to 0.33% in the concurrent vehicle control (not statistically significant). At 2000 mg/kg, the mean numerical aberration frequency was 0.2% compared with 0.7% for the concurrent vehicle control. Frequencies of cells with numerical aberrations at 2000 mg/kg fell within the historical vehicle control 95% reference range.

***Table**: Summary of results of cells with structural aberrations (excluding gaps) following exposure to pydiflumetofen in the Wistar rat.*

16 Hr data:

Treatment (mg/kg)	Cytotoxicity (%) $	Cells Scored	Aberrant Cells Excluding Gaps*	Aberrant Cells Excluding Gaps (%)	Fisher's Exact Test	Statistical Significance
Vehicle	-	1200	5	0.42	-	-
500	6	1200	5	0.42	0.500	NS
1000	24	1183	5	0.42	0.491	NS
2000	27	1114	8	0.72	0.175	NS
CPA, 30	78	554	165	29.78	0.000	p≤0.001

* Historical vehicle control 95% reference range (excluding gaps) range 0 to 0.5%
$ Cytotoxicity based on mitotic inhibition

42 Hr data:

Treatment (mg/kg)	Cytotoxicity (%) $	Cells Scored	Aberrant Cells Excluding Gaps*	Aberrant Cells Excluding Gaps (%)	Fisher's Exact Test	Statistical Significance
Vehicle	-	1200	4	0.33	-	-
2000	17	1200	7	0.58	0.193	NS

* Historical vehicle control 95% reference range (excluding gaps) 0 to 1.03%
$ Cytotoxicity based on mitotic inhibition

Cyclophosphamide (30 mg/kg) was used as a positive control. At the 16 hour sample time, the mean percentage of cells with aberrations (excluding gaps) was 29.8%.

Several pharmacokinetic studies in the rat were evaluated in the plant protection DAR. In a preliminary pharmacokinetics study (DAR B.6.1.1; *Anon, 2015*; K-CA 5.1.1/01), at a dose of 1000 mg/kg, pydiflumetofen and its metabolites were observed in blood over a 72 post-dose period. It may thus be concluded that the bone marrow was exposed to pydiflumetofen and its metabolites at doses of 500, 1000 or 2000 mg/kg prior to the 16 hour sampling point and 2000 mg/kg prior to the 42 hour sampling point in the chromosome aberration test.

RAC concludes that pydiflumetofen did not induce chromosome aberrations in the bone marrow cells of male rats treated up to 2000 mg/kg.

Conclusion

The *in vitro* mutagenicity tests were negative. There was limited evidence that pydiflumetofen induced structural chromosomal aberrations in an *in vitro* clastogenicity study in human lymphocytes, RAC considers the study equivocal. The *in vivo* mouse bone marrow micronucleus tests were negative, the test substance did not induce micronuclei and pydiflumetofen is considered to be non-mutagenic in this assay. Furthermore, in an *in vivo* rat bone marrow

chromosome aberration assay, pydiflumetofen was negative in male rats treated up to 2000 mg/kg.

Classification Assessment

Muta 1

No human data are available for pydiflumetofen, therefore a classification with Muta. 1A is not supported. Pydiflumetofen is negative in acceptable *in vitro* tests and negative in *in vivo* somatic cell mutagenicity guideline tests in mammals. Data are not available illustrating the induction of mutagenic effects in germ cells (a criterion for Category 1B). RAC does not support classification with Muta. 1A or B.

Muta 2 Assessment and conclusion

The overall weight of evidence for pydiflumetofen supports no potential for genotoxicity in somatic cells from a battery of *in-vivo* and *in-vitro* GLP and guideline compliant studies. Therefore no classification in category 2 is warranted.

RAC agrees with the DS that **no classification of pydiflumetofen for genotoxicity is warranted**.

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

Two guideline and GLP compliant long-term oral (dietary) toxicity/carcinogenicity studies were available to the DS: a 2-year combined chronic toxicity/carcinogenicity study in the Han Wistar rat (*Anonymous, 2015a*) and an 18-month carcinogenicity study in the CD-1 mouse (*Anonymous, 2015b*). Study details were summarised in Table 38 in the CLH report. Pydiflumetofen had no treatment-related neoplastic findings in rats. However, in mice there was a treatment related increased incidence of hepatocellular carcinomas and adenomas (present as multiple adenomas) observed in males at 45 and 288 mg/kg/day, which were statistically significant at the high dose only. There were no treatment-related neoplastic findings in females at similar doses in this study. Several additional studies were conducted to investigate the Mode of Action (MoA) and human health relevance of the rodent tumours. The DS concluded from this data that pydiflumetofen did not meet the criteria for classification and therefore no classification for carcinogenicity was proposed.

1. In-vivo animal studies

1.1 Rat 2-year dietary toxicity/oncogenicity study

In a rat GLP and OECD TG 453 (2009) compliant carcinogenicity dietary study (*Anonymous, 2015a*), treatment with pydiflumetofen did not reduce the survival of rats up to the highest doses tested. Crl:WI (Han) strain rats were divided into treatment groups and scheduled kills were conducted after 12 months treatment for 12 animals/sex/group and at study termination after 24 months treatment for 52 animals/sex/group.

***Table**: Mean dose received (mg/kg/day)*

Dietary concentration of pydiflumetofen (M/F ppm)	0	200/ 150	1000/ 450	6000/ 1500
Males	0	9.9/	51/	319/
Females	0	10.2	31.0	102

Dose level selection was based on pharmacokinetic data. The DS did not explain the rationale behind the dose selection. They only noted the concern raised in the DAR on whether the doses were high enough to account for the presence of the primary metabolite 2,4,6 trichlorophenol (2,4,6-TCP) which has a Carcinogenicity Category 2 classification in Annex VI to CLP.

General toxicity was displayed by significantly lower body weight (9-18%), body weight gain (13-22%) and slight reductions in food consumption which were observed in both sexes at the mid and top dose (51 and 319 mg/kg/day in males; 31 and 102 mg/kg/day in females). The liver was clearly a target organ, with slight effects also observed in the thyroid. The effects observed included increased liver weight and hepatocellular hypertrophy associated with cytoplasmic eosinophilic inclusions, and small increases in incidence of thyroid follicular cell hyperplasia.

Organ weight changes considered to be treatment related were observed in the liver. There were no treatment related differences in the functional observation battery parameters following administration of pydiflumetofen. There were no treatment-related neoplastic findings at the 12-month interim sacrifice.

1.1.1 Neoplastic findings

According to the CLH report there was no evidence of an increase in liver neoplastic lesions or early onset of neoplasms or other potential carcinogenic findings following exposure to pydiflumetofen at doses up to 319 and 102 mg/kg bw/day in males and females, respectively.

In the response to comments document following public consultation of the CLH report, the DS acknowledged that an increase in the incidence of thyroid follicular cell adenomas was observed at the highest tested dose of 102 mg/kg/day in females (3/51 (5.9%) *vs* 1/51 (2%) in the control group). The DS noted a re-evaluation of the historical control data and submission of additional information from the applicant following peer review under Regulation 1107/2009. They concluded that the increase in the incidence of thyroid follicular cell adenomas was not treatment related because they fell within the revised historical control data (7 studies; 2009-2015, background incidence of 0-5.8%; DAR table 6.5-16).

The DS also noted that although it considered the dose levels selected for the long term/carcinogenicity study in rat to be low, it also concluded that the maximum tolerated dose (MTD) had indeed been reached. This was in line with many published technical guidelines for chronic or lifetime bioassays. Significant treatment-related decreases were observed in body weight gains in high dose males (↓18%) and high dose females (↓13%). Additionally, significant increases were observed in males for absolute and relative liver weights, which were associated with hepatocellular hypertrophy in both sexes and liver eosinophilic inclusions (a non-neoplastic lesion according to the US NTP Non-neoplastic Lesion Atlas).

1.2 Mouse 18-month dietary carcinogenicity study

In a mouse GLP and OECD TG 451 (2009) compliant carcinogenicity dietary study (*Anonymous, 2015b*), treatment with pydiflumetofen did not reduce the survival of mice up to the highest doses tested. Groups of 50 male and 50 female CD-1 mice were fed diets containing 0, 75, 375 or 2250 ppm of pydiflumetofen for a period of at least 80 weeks.

Table: *Mean dose received (mg/kg/day)*

Dietary concentration of pydiflumetofen (ppm)	0	75	375	2250
Males	0	9.2	45.4	287.9
Females	0	9.7	48.4	306.2

General toxicity was limited, but a small effect was observed on mean body weight in males and females treated at the high dose. The magnitude of the differences in body weight compared to controls was moderate after 80 weeks (-7% in males; -12% in females), indicating that there was no exacerbating systemic toxicity. This was associated with a statistically lower group mean body weight gain compared to the control animals and lower food consumption. Food utilization was only significantly lower in males at the high dose.

Liver weights were increased in both sexes at the high dose. Males showed an enhanced sensitivity to treatment with absolute and relative liver weights increased by 37% and 47% respectively over controls, compared with females showing increases of 9% and 26% respectively over controls. Prominent lobular architecture (seen in the rat) was not a feature of treatment in mice. Centrilobular hypertrophy was observed in males only at 45 and 288 mg/kg/day. There was an increasing incidence of eosinophilic foci of cellular alteration in the liver of male mice with indications of a dose response relationship. The incidence was statistically significant at the top dose of 288 mg/kg/day and historical controls were exceeded at ≥ 45 mg/kg/day. The DS concluded that the slightly higher incidence of eosinophilic foci of cellular alteration in the liver of male mice at 45 mg/kg/day was incidental to treatment.

1.2.1 Neoplastic findings

No neoplastic findings were observed in female mice.

A higher incidence of hepatocellular carcinomas and adenomas was observed in males administered pydiflumetofen at 45 and 288 mg/kg/day compared with the control group. These correlated with liver masses observed at necropsy. Hepatocellular adenomas and carcinomas in treated groups were only detected at terminal necropsy, i.e. there was no indication of a reduction in tumour latency. While the incidences of hepatocellular adenomas ($p \leq 0.01$) and carcinomas ($p \leq 0.05$) were statistically significantly increased in the top dose group, the increased incidence at 45 mg/kg/day was also considered to be treatment-related due to the number of animals with multiple hepatocellular adenomas in the liver. A clear positive dose response relationship was evident for (1) incidences of animals with tumours and (2) incidence of animals with multiple tumours.

2. Mechanism of action and supporting data relevant for findings in the mouse liver

2.1 Description and results from the mechanistic studies

The DS briefly described several mechanistic studies (table 40 of the CLH report) that were conducted to elucidate the mode of action (MOA) for the liver hepatocellular carcinomas and adenomas observed in male mice. However, several alternative possible mechanistic explanations were not investigated in detail. Enzyme analysis of the liver indicated no to low involvement of peroxisome proliferation and some minor increase in Cyp1a activity. The available investigations focused on providing evidence in support of one mode of action, i.e. on a non-genotoxic mode of action involving hepatocyte proliferation, induced via constitutive androstane receptor (CAR) activation.

The DS summarised the available studies in a table which included:

- 1 x *in-vitro* CAR reporter gene assay (*Omiecinski, 2014*).
- 1 x *in-vivo* 28-day mouse dietary studies for evaluation of liver effects (*Elcombe, 2015*).
- 1 x *in-vitro* study with mouse hepatocytes (*Lowes, 2015a*)
- 1 x *in-vitro* study with human hepatocytes (*Lowes, 2015b*).
- 1 x *ex-vivo* 28-day dietary mouse hepatocellular proliferation/enzyme induction study (*Haines, 2012*).

There were no *in-vivo* studies with CAR-knock out rodents for confirmation of CAR mediated effects.

The *in vitro* CAR activation assay confirmed pydiflumetofen was a direct activator of CAR from the mouse, rat and human, and that the CAR constructs from the different species were efficacious in that they were all functionally expressed in COS-1 cells (monkey kidney immortal fibroblast cell line), although the human constructs showed lower activity than those from rodents.

The *in vivo* 28-day male mouse dietary study for investigation of liver effects confirmed (at the highest dose, 324 mg/kg/day), increased liver weights (↑ 22-24%), which were accompanied by hepatocellular hypertrophy, increased cytochrome P450 levels, and increased PROD activity after 2, 7 and 28 days of treatment. In addition, increased DNA-synthesis (S-phase) was maximal after 2 days of treatment and was accompanied by a histopathology finding of increased mitosis.

The *in vitro* study with male CD-1 mouse hepatocytes resulted in increased cell proliferation (S-phase of the cell cycle) and in initially increased PROD and BROD activities (CYP2B/CYP3A expression).

The *in vitro* study with male human hepatocytes (from a single donor) had no effect on cell proliferation (S-phase of the cell cycle). CYP2B/3A activities (measured as PROD and BROD activities) were elevated.

The *ex vivo* 28-day mouse dietary study focused on enzyme analysis of liver samples. The study was designed to evaluate liver peroxisomal and microsomal enzyme expression in male and female mice on days 3, 7 and 28-days following exposure to pydiflumetofen at up to 7000 ppm (dose in mg/kg not reported).

- CN^--insensitive palmitoyl CoA oxidase: slight suppression → no evidence for enhanced hepatic peroxisomal β-oxidation.
- total cytochrome P450 content (1.2 – 1.8-fold vs controls, day-28): dose-related increase in hepatic total P450 content
- EROD activity: weak effect (1.3 – 1.4-fold vs controls, 28-days) → no evidence for significant CYP1A expression. There was an initial burst of activity on days 3 and 7 (2-3 fold increase relative to control).
- PROD: substantial and significant enhancement of activity (9 – 15-fold in males, [about 3-5-fold in females] vs controls, 28-days) → evidence of strong CYP2B enhanced expression.
- BQ: small enhancement of enzyme activity (1.5 – 3.5-fold vs controls, day-28) → evidence for limited CYP3A enhanced expression.
- Lauric acid 12-hydroxylation (LAH): weak enhancement of enzyme activity in males only (1.3 – 3.5-fold vs controls, day-28) → evidence for limited CYP4A activity

The study showed liver enzyme effects consistent with a typical CAR/PXR activator; a dose-dependent increase of total cytochrome P450 content (max. 1.8x), PROD (max. 15x) in males, max. 5x in females), and BQ (max. 3.5x in males, max. 2.5x in females) was observed. AhR involvement was shown to be weak (no significant EROD activity). There was limited evidence for a small increase in expression of CYP4A which suggests some limited involvement of PPARα activation though there was no clear evidence for enhanced hepatic peroxisomal β-oxidation.

Inhibition of apoptosis and other associative events in the CAR associated tumour model have not been investigated (e.g. altered epigenetic changes, gap junctional intercellular communication and oxidative stress).

The available mechanistic data indicate that the MoA for liver tumours in mice is supportive of hepatocellular proliferation induced by activation of the CAR.

2.2 Conclusions

The evaluation by the DS of mode of action studies against the IPCS and ILSI/HESI framework demonstrated a CAR activation MOA. Data generated in human hepatocytes indicated human non-relevance due to quantitative differences in responses to proliferative stimuli

3. Mechanism of action and supporting data relevant for neoplastic findings in the female rat thyroid

3.1 Description and results from the mechanistic studies

The DS briefly referenced two mechanistic studies (table 40 of the CLH report) that were conducted to clarify the mode of action (MOA) for the slight numerical increase in the incidence of thyroid follicular cell adenomas (only in the female rat), at the top dose of 102 mg/kg/day. The incidence of adenomas did not reach statistical significance on pairwise comparison or show a dose-related trend. There was no discussion about these studies in the CLH report, only in the DAR:

- 1 x *in vitro* thyroid peroxidase activity in rats (*Lake, 2014*).
- 1 x *ex vivo* hepatic microsomal UDP-GT activity in livers of male rats (*Lake, 2015*). Samples derived from male rats on 90-day dietary study.

The aim of the *in vitro* thyroid peroxidase activity study was to evaluate the effect of pydiflumetofen (0, 0.007, 0.1, 1.5 and 10 µM) on rat thyroid peroxidase activity *in vitro*. A pooled thyroid gland microsomal preparation from five male rats was assayed for thyroid peroxidase activity by determining the monoiodination of L-tyrosine. The positive control, 6-propyl-2-thiouracil (PTU; 10 µM) resulted in a 99.9% inhibition of thyroid peroxidase activity. Pydiflumetofen had no effect on rat thyroid peroxidase activity at any concentration tested.

The *ex vivo* hepatic microsomal UDP-GT activity study was designed to evaluate the effect of treatment with pydiflumetofen on hepatic microsomal UDP-glucuronosyl transferase activity. Male rat liver samples were taken at termination of the 90-day dietary toxicity study where the rats were given diets containing 0, 18.6, 111, and 587 mg/kg/day active substance. Liver samples were not taken from the top dose group (16000 ppm or 1187 mg/kg/day).

Hepatic microsomal UDP-glucuronosyl transferase activity (using thyroxine as substrate), expressed as (i) specific activity, (ii) per gram of liver, (iii) per total liver and (iv) per relative liver weight, was significantly ($p < 0.01$) increased to 171, 194, 224 and 239% of control, respectively, by treatment with 111 mg/kg/day pydiflumetofen and to 288, 347, 421 and 486% of control, respectively, by treatment with 587 mg/kg/day pydiflumetofen ($p < 0.01$). Female livers were not tested.

3.2 Conclusion

The RMS concluded that pydiflumetofen was a positive inducer of rat hepatic microsomal UDP-glucuronosyl transferase activity as determined from assays that used thyroxine as substrate.

4. Consideration of the dose level selection in the toxicity studies and the carcinogenic potential and impact of 2,4,6 TCP

4.1 Background

The DS described uncertainties expressed in the DAR regarding the dose level selection employed in some of the toxicity studies based on pharmacokinetic data (see section 2.6.1 and B.6.1 in volume 3). They were critical of the additional pharmacokinetic studies that investigated the TK profile of the active substance with non-radiolabelled pydiflumetofen following single or repeated doses. Pydiflumetofen was extensively and rapidly metabolised and without an appropriate radiolabel it was not possible to follow the fate of several important metabolites including 2,4,6-trichlorophenol (2,4,6 TCP). The dose selection arguments put forward by the applicant did not consider one of the major circulating metabolites following administration of pydiflumetofen, i.e. 2,4,6 TCP. Its plasma concentration largely exceeded that of the parent. It would have been appropriate to also investigate the pharmacokinetics of 2,4,6 TCP following repeated or single oral administration of pydiflumetofen, especially since this metabolite is of toxicological concern (classified as Carc 2. H351, by the European Union, and similarly by other international regulatory bodies).

The applicant suggested that as the dose increased, the fraction of pydiflumetofen absorbed decreased and therefore systemic levels of metabolites were presumed to also not increase. The kinetic studies showed that systemic exposure to pydiflumetofen increased in an approximately proportional manner after both single and repeated doses up to 30 mg/kg (with oral absorption remaining constant at around 85-90% of the dose). Between 30 and 100 mg/kg systemic exposure also increased but in a non dose-proportional manner. As the dose increased beyond 100 mg/kg (oral gavage), absorption became limited, with < 55% of a 100 mg/kg dose to females and < 24% of a 300 mg/kg dose to males absorbed. At these doses, unchanged pydiflumetofen was the major component in faeces which contained up to 63% of the dose, but less than 0.2% was detected in bile. According to the applicant, the fraction or amount of dose absorbed approaches equivalence and so even if the nominal dose was increased the systemic dose was effectively the same. At very high doses, absorption was assumed not to be influenced by repeated administration and therefore systemic levels of metabolites would not be expected to increase.

The DS and RMS reserved most concern for the dose levels selected by the applicant for the long-term and reproductive toxicity studies. The applicant justified their dose selection based on TK data and dose limited absorption, i.e. with higher oral doses, less compound was absorbed into the blood. However, this argument was regarded as insufficient. This does not mean that systemic exposure does not continue to increase with doses higher than the highest dose levels selected by the applicant for the long-term and reproductive toxicity studies. On the contrary, comparison between plasma AUCs determined after administration in rats of the phenyl radiolabelled active substance at dose levels up to 1000 mg/kg/day, showed that systemic exposure still increased beyond 100 or 300 mg/kg/day:

1. by 4-fold between 100 - 1000 mg/kg bw/d
2. and by 1.7-fold between 300 - 1000 mg/kg bw/d

Also, increased systemic exposure at very high doses was confirmed by the short-term repeated dose studies where an increase in toxicity (liver and body weight effects) was observed with increasing doses beyond the maximal doses selected by the applicant for the long-term or reproductive toxicity studies.

The DS and RMS believed the doses of pydiflumetofen chosen for the long-term studies might not have been sufficiently high to cover the carcinogenic potential of 2,4,6 TCP in the rat. High systemic exposures of pydiflumetofen (and consequently 2,4,6 TCP) resulting from

administration of doses higher than 300 mg/kg (males) have not been tested in the rat long-term study. Furthermore, female rats were only dosed up to 102 mg/kg, at which concerns for thyroid adenomas were already expressed in the 2-year rat dietary study.

Leukaemias were observed from an estimated dose of 250 mg/kg/day of 2,4,6 TCP in male rats in a long-term toxicity study from the NTP (NCI, 1979; see Additional key elements for a summary of this study). *Note*: it is not clear how this dose was calculated since no food consumption data was recorded for the original NCI study.

In the CLH report (2018) for pydiflumetofen, no tumours were observed following 2-year administration in male rats at a dose of up to 300 mg/kg/day. The DS believed that the maximum tolerated dose (MTD) was reached in the 2-year study in rats as the top dose in males (300 mg/kg/d) resulted in an 18% reduction of body weight. They considered that the carcinogenic potential of pydiflumetofen was thus appropriately assessed. To verify whether higher doses of pydiflumetofen (>300 and up to 1000 mg/kg bw/day) would have covered the carcinogenic potential of 2,4,6 TCP, a risk analysis was performed by the RMS to estimate systemic exposure to 2,4,6 TCP. This considered the oral absorption of pydiflumetofen and the proportion of 2,4,6 TCP measured in plasma (% of AUC) after an oral administration of radiolabeled pydiflumetofen. The DS concluded that the higher tested dose of 300 mg/kg bw/d of pydiflumetofen in the long term rat study was actually not sufficiently high to elicit the carcinogenic potential of its major circulating metabolite 2,4,6 TCP (DAR, B.6.8.1.6, p614). They go on to state that a dose of about 1000 mg/kg/day would be required. They then further stated that the mammalian toxicity data package on pydiflumetofen had sufficiently assessed the toxicity of 2,4,6 TCP and its conjugates, but they did not explicitly state that the carcinogenicity of the metabolite has been adequately assessed.

4.2. Conclusions

It is clear that the RMS (as expressed in the DAR) had doubts over whether pydiflumetofen had been adequately tested in a high enough dose in the long-term or reproductive toxicity studies. They concluded on one hand that the highest dose (for males) in the long term rat study was actually not sufficiently high to elicit the carcinogenic potential of the metabolite 2,4,6 TCP; while on the other hand stating that the MTD was achieved and that the mammalian toxicity data package on pydiflumetofen had sufficiently assessed the toxicity of 2,4,6 TCP and its conjugates. It was not a very clear conclusion by the DS/RMS.

Comments received during public consultation

Two MSCA submitted comments.

Comment 1

This MSCA supported classification of pydiflumetofen with Carc 2, H351 based on a significant increase in liver cell adenoma and carcinoma in high dose male mice. They accepted a MoA involving CAR in the mouse but the lack of tumourigenicity in rats if the same MoA was operating was problematic. This could be due to (i) further modes of action of unknown human relevance are involved in tumour formation in male mice or (ii) the absence of a similar neoplastic effect in the rat might be due to the rather low dose levels (up to 100 or 300 mg/kg bw/day in female/male rats) employed in the long-term study. The MSCA were not convinced that the MTD was reached.

The DS responded that it considered that the MTD was reached. Significant treatment-related decreases were observed in body weight gains in high dose males (↓ 18%) and high dose females (↓ 13%). The DS agreed that the major metabolite 2,4,6-TCP, must be taken into consideration. The DS calculated that a pydiflumetofen dose of 1000 mg/kg bw/day could potentially give rise

to a systemic exposure of TCP which might account for 25% of leukaemias observed in the rat (T25) based on the NTP study data performed with 2,4,6-TCP (NCI 1979). The relevance of this risk based assessment was questioned by RAC. The DS agreed with the proposed MoA leading to liver tumours in mice (through CAR activation) and that sufficient data was provided by mechanistic studies. The DS also mentioned concern regarding the use of SDHI (succinate dehydrogenase inhibitor) fungicides in agriculture.

Comment 2

The second MSCA commented with respect to the rat thyroid adenomas and the increase in eosinophilic foci of cellular alteration in the liver of male mice from the long-term studies and their relevance in setting hazard-based endpoints such as NOAELs. They considered the thyroid adenomas to lie outside the HCD and be treatment related in the rat. They considered the preneoplastic foci to be treatment related at the 75 ppm (9.2 mg/kg/day) dose in male mice.

The DS responded that following peer review the most relevant HCD within a 5 year timeframe gave a background incidence of 0-5.8% (3/52) (as presented in Table 6.5-16 in the revised DAR) for rat thyroid adenomas. The increase in the incidence of thyroid follicular cell adenomas (3/51 (5.9%) vs 1/51 (2%) in control), observed at the highest tested dose for females, 1500 ppm (102 mg/kg/day), can be considered to be within the historical control data and not treatment related. In support of this they noted there were no preneoplastic lesions (hyperplasia) observed at 12 or 24 months in the long term study and no histopathological findings were observed in thyroids from females in other rat toxicity studies (28/90 days and 2 generation reproductive toxicity). Mechanistic data had shown that pydiflumetofen does not have a direct effect on thyroid peroxidase in the rat (*in vitro*) and therefore, pydiflumetofen was not acting via a direct effect on the thyroid.

The DS explained that during the peer review process under Regulation 1107/2009, new HCD for eosinophilic foci of cellular alteration in the liver of male mice from long-term studies was received (2007 – 2013, a total of nine 80-week mouse studies). This new data showed that the incidence of eosinophilic foci at 75 ppm (9.2 mg/kg/day equivalent dosing and incidence of 8%) was still outside the revised HCD range (0-6%). The DS noted that the increase in preneoplastic foci at the 9.2 mg/kg/day dose was not statistically significant; they occurred in terminal animals only, indicating there was no reduction in latency; and eosinophilic foci were not observed in the 90 day mouse study where only an increase in hepatic centrilobular hypertrophy was observed in the absence of hepatic necrosis. Taking several other factors into account, the DS did not consider this effect at 9.2 mg/kg/day to be treatment related and that a NOAEL could be set at 9.2 mg/kg/day.

During the second RAC member consultation of the opinion for pydyflumetofen which was opened some time before RAC-48, industry submitted three position papers regarding the adequacy of dose selection, the liver carcinogenic response in male mice and the delay in sexual maturation in rats in the 2-generation study.

The position paper on carcinogenicity was entitled - Company "Comments: Pydiflumetofen - Mechanism of Mouse Liver Carcinogenicity and Non-Relevance to Man". They concluded "*The mechanism by which Pydiflumetofen causes liver tumours in the male mouse has been clearly demonstrated as mediated by the constitutive androstane receptor (CAR). The key event in this MoA has been demonstrated to not occur in human. All relevant alternative MoAs have been excluded*". No new data was presented in this report. It outlined the work that was done and how the available data fitted with the CAR MoA to produce tumours in mice.

The position paper on dose selection was entitled – Company "Comments: Pydiflumetofen – Adequacy of Dosing in Toxicology Studies. The company concluded that "*Systemic exposure to pydiflumetofen becomes limited by absorption as the external dose increases, until there is no*

appreciable increase in internal exposure. At doses above the linear range of toxicokinetic exposure, the inherent hazard of the chemical cannot be assessed, as large quantities of unabsorbed test item residing in the GI tract may impact on food transit time and nutrient absorption, disrupting normal biological homoeostasis. Therefore, adverse effects observed at doses exceeding the inflexion point of linear kinetics are not related to the test-item, but may be related to biological stresses and not relevant to hazard characterisation. Therefore, based on the non-linear kinetics through absorption limited exposure, which has clearly been demonstrated for individual animals, studies have been more than adequately dosed (as the high doses were actually set above the point where linearity is lost and the inherent hazard of pydiflumetofen has been fully assessed".

[5. Additional key elements in BD]

Assessment and comparison with the classification criteria

6. Carcinogenicity

6.1 Introduction

Pydiflumetofen induced liver tumours in mice and thyroid tumours in rats, thus there is a need to consider whether classification for carcinogenicity is appropriate. There is no information from studies in humans to inform on carcinogenic potential and so classification in category 1A may be excluded from further consideration.

6.2 Rat thyroid tumours

In females there was a slight numerical increase (3/51 (5.9%) vs 1/51 (2%) in control) in the incidence of thyroid follicular cell adenomas at 102 mg/kg/day (1500 ppm), which did not reach statistical significance on pairwise comparison, or show a dose-related trend, and was at the upper bound limit of the most relevant historical control range of 0-5.8% (table below). There were no recorded thyroid follicular cell carcinomas at any dose. There was no substance-related effect on the incidence of C-cell tumours. There were no preneoplastic lesions observed. Some non-neoplastic microscopic findings were present. The incidence of 3/51 (5.9%) observed for follicular cell hyperplasia in females at the highest dose of 102 mg/kg/day was within the historical control data (range: 0- 6%). These findings are considered possibly related to administration of pydiflumetofen but are not sufficient for classification.

Table: *Summary of thyroid neoplastic findings in female rats*

Finding	**Dose Concentration (mg/kg/day) Females**				
	0	**10.2**	**31.0**	**102**	**Historical control data#**
Thyroids (no. examined)	51	52	51	51	439
Follicular cell adenoma	1 (2%)	0	0	3 (5.9%)	9 (2%) range 0-5.8%
Hyperplasia; Focal, Follicular cell	1 (2%)	3 (5.8%)	0	3 (5.9%)	12/323 (3.7%) range 0-6%
Thyroid masses at necropsy	0	0	0	2	--

Note: thyroid follicular cell adenomas were also present in males but their incidences at all dose levels was below the concurrent controls.

Regarding the metabolite 2,4,6-TCP, there is no data to indicate that a sufficiently high dose of parent active substance was tested in the long-term study to elicit the carcinogenic potential of one of the major circulating metabolites. The carcinogenicity of this metabolite has not been adequately assessed in either the long-term rat or mouse studies. The RMS estimated that a limit dose of 1000 mg/kg/day pydiflumetofen would have been required to at least reach levels of 2,4,6-TCP sufficient to elicit a tumourigenic response comparable to 25% that observed in male rats in the NTP study with 2,4,6-TCP[1]. Whether such information is relevant to a classification proposal for pydiflumetofen needs to be considered. With the available data and the maximum tested dose of approximately 300 mg/kg/day in males there was no indication of a concern for leukaemia in the tested animals (the major carcinogenic effect of 2,4,6-TCP in male F344 rats). The raw data from the long-term feeding study for pydiflumetofen showed no evidence of leukaemia or perturbations of white blood cell and lymphocyte counts.

Two mechanistic studies were performed investigating effects on the thyroid (table below). A mechanistic study on increased phase II metabolic activity in the livers of male rats indicated an increase of approximately 2-5 fold over concurrent controls in the activity of microsomal UDP-glucuronosyl transferase (at doses of 111 and 587 mg/kg/day over 90-days). This may account for increased thyroxine clearance by the liver and place the thyroid under secondary feedback pressure to increase T4 output and alter the expected incidence of thyroid follicular tumours. However, the incidence of follicular cell adenomas in males in the top dose group did not exceed those in the concurrent controls. A major weakness of the study is that it did not investigate UDP-glucuronosyl transferase activity from female rat livers. Females were the more sensitive sex and ideally the mechanistic investigation should have been performed with female livers rather than those from males. A second mechanistic study (males only), found that pydiflumetofen had no direct effect on rat thyroid peroxidase activity at any concentration tested.

RAC can conclude on a classification only based on an assessment of all the data that has been presented in the available studies. In this context, the rat thyroid adenomas occur at the upper boundary limit of the HCD range. There was no supporting evidence from males that pydiflumetofen elicits a tumourigenic response. Thyroid tumours were not observed in mice. RAC notes that it is regrettable that a higher concentration was not investigated in the female rat and also notes that female livers were not analysed for increased phase II metabolic activity. RAC concludes that there is no firm evidence from the available data for a neoplastic response in the rat thyroid as a consequence of exposure to pydiflumetofen.

[1]National Cancer Institute. (1979). Bioassay of 2,4,6-Trichlorophenol for Possible Carcinogenicity. National Cancer Institute Technical Report Series No. 155, 1979. National Institutes of Health, Bethesda, Maryland 20014, USA.

Table: *RAC Summary of mode of action studies investigating effects on the rat thyroid*

Endpoints investigated	Summary observations	Reference
1. In-vitro thyroid peroxidase activity. - microsomal preparation from five male rats - Table 40 CLH report	1. Pydiflumetofen had no effect on rat thyroid peroxidase activity.	Lake, (2014)
2. Ex-vivo 90-day rat oral (dietary) study - (Crl:WI(Han) strain - Male liver samples - Pydiflumetofen tested (0, 18.6, 111 and 587 mg/kg/day) - Total cytochrome P450 levels - Enzymes: hepatic microsomal UDP-GT activity - Table 40 CLH report	**a. Enzyme activity:** 1. Pydiflumetofen induced substantial hepatic microsomal UDP-GT activity (2-5 fold relative to control). **b. Hepatic microsomal protein:** 1. ↑ hepatic microsomal protein content; 114 and 122% of control at the two top doses.	Lake, (2015)

6.3 Mouse liver tumours

In animals sacrificed at 18 months, an increased incidence of hepatocellular carcinomas and adenomas was observed in <u>males</u> administered pydiflumetofen at 45 and 288 mg/kg/day compared with the control group (table below). In animals that received ≥ 45 mg/kg/day a clear increase in multiplicity of tumours was observed. <u>No neoplastic findings were observed in female mice</u>. No increases in liver tumours were noted in the rat lifetime study.

Table: *Summary of hepatocellular neoplastic findings in the liver in males*

Finding	Dose Concentration (mg/kg/day) Males				
	0	**9.2**	**45.4**	**288**	**Historical control data[#]**
Liver (no. examined)	50	50	49	50	250
hepatocellular carcinoma [multiple]	2 (4%) [0]	3 (6%) [0]	4 (8.2%) [0]	10* (20.0%) [2]	19 (7.6%) range 6-10% [3-5/250]
hepatocellular adenoma [multiple]	4 (8.0%) [0]	6 (12.0%) [0]	9 (18.4%) [7]	22** (44.0%) [14]**	45 (18.0%) range 10-28% [5-14/250]
Liver masses at necropsy	5	9	14	20	

* Statistically significant difference from control group mean, $p<0.05$ (Fisher's exact test)

** Statistically significant difference from control group mean, $p<0.01$(Fisher's exact test)

[#] Incidence range (min-max). HCD from five 80-week carcinogenicity in mice performed by the conducting laboratory Charles River Edinburgh between 2007 and 2009.

In support of the findings in liver, hepatocellular carcinomas and adenomas correlated with liver masses observed at necropsy at ≥ 45 mg/kg/day. Pre-neoplastic lesions were apparent. There was an increasing numerical trend for a higher incidence of eosinophilic foci of cellular alteration in the livers of males with increasing dose (table below). The incidence was only significantly higher (Fisher's exact test) in males dosed at 288 mg/kg/day compared to the control group.

Table: *Comparison of histopathology findings in males (including presumptive preneoplastic lesions)*

Finding		**Dose Concentration (mg/kg/day) Males**				
		0	**9.2**	**45.4**	**288**	**HCD**
Liver (no. examined)		50	50	49	50	
hepatocellular hypertrophy:	total	0	0	6*	18**	
	minimal	0	0	2	2	
	mild	0	0	3	10	
	moderate	0	0	1	6	
focus of cellular alteration, eosinophilic	total	1	4	6	10**	7/250 (2.8%)
	minimal	0	0	1	1	Range: 0 – 6%
	mild	1	2	1	3	
	moderate	0	2	4	6	

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

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

In summary, pydiflumetofen was considered to cause carcinogenic effects in the livers of male mice that received ≥ 45.4 mg/kg/day where higher incidences of liver tumours (hepatocellular carcinomas and adenomas (multiple)) were observed. A clear dose response relationship was evident.

6.3.1 Mouse liver tumours: assessment of the Mode of Action

The tumour profile observed in pydiflumetofen carcinogenicity bioassays was typical of a non-genotoxic mechanism (single species, single sex and single organ involvement without decreased latency). MoAs involving induction of other CYP P450 isoforms or peroxisome proliferation may be excluded based on the nature of the liver findings. Increased liver weight and hepatocellular hypertrophy are not specific surrogate markers for CAR activation because the induction of other CYP P450 isoforms or peroxisome proliferation can also produce these findings. However, these other MoAs can be ruled out because the experimental evidence showed that pydiflumetofen treatment did not result in any significant biochemical evidence for peroxisome proliferation within the liver hepatocytes (e.g. no ↑ CN^--insensitive palmitoyl CoA oxidase, and limited ↑ LAH). Hepatocellular cytotoxicity and subsequent regenerative proliferation, such as that caused by chloroform, is another mechanism by which carcinogenesis can occur. This mechanism is typically characterised by sustained diffuse necrosis and cellular proliferation. In this case it can be excluded for pydiflumetofen because the data from the i*n vivo* studies demonstrated a lack of hepatic damage and regenerative proliferation at all time points investigated.

There are various possible mechanistic explanations that can be considered for the carcinogenic response in mice and a limited investigation into these other modes of action was undertaken, which may be summarised as follows:

- genotoxicity → negative data in this case → conclusion: unlikely
- cytotoxicity → the liver was the target organ but there were no indications from histopathology to support this as a primary MoA. The incidences of focal/ multifocal necrosis and focal/ multifocal vacuolation were zero to low and similar across all

treatment groups, or similar to controls. However, *in vitro* tests with human hepatocytes did show increased sensitivity relative to mouse hepatocytes, so the question regarding cytotoxicity as a factor still remains unresolved.

- PPARα receptor activation → limited to no effect in this case → conclusion: unlikely
- CAR/PXR receptor activation → positive data in this case → conclusion: plausible
- AhR receptor activation → very limited effect → conclusion: unlikely as a primary mechanism, possible crosstalk between receptors or limited induction of Cyp1A/AhR
- Porphyria → no data
- Endocrine mediated proliferation → no data, no evidence from other studies.
- Immunosuppression → no data

Recognising that pydiflumetofen may be associated with a hepatocarcinogenic effect in mice, the applicant sponsored a series of mechanistic studies to investigate a possible non-genotoxic mode of action involving liver stimulation via constitutive androstane receptor (CAR) induction. The DS presented these studies and others from the plant protection DAR. Some of the key and associative events in this process are:

- CAR activation
- Altered gene expression specific to CAR activation
- Increased cell proliferation
- Inhibition of apoptosis
- Clonal expansion leading to altered foci
- Liver adenomas/carcinomas

Such a non-genotoxic mode of action leading to liver tumour formation in rodents has been considered of limited to no relevance to humans. The mechanistic data presented in the CLH report and the DAR suggests that the CAR activation model (table below) is the most plausible. The data from pydiflumetofen support a proposed MoA in male CD-1 mice involving the following key events:

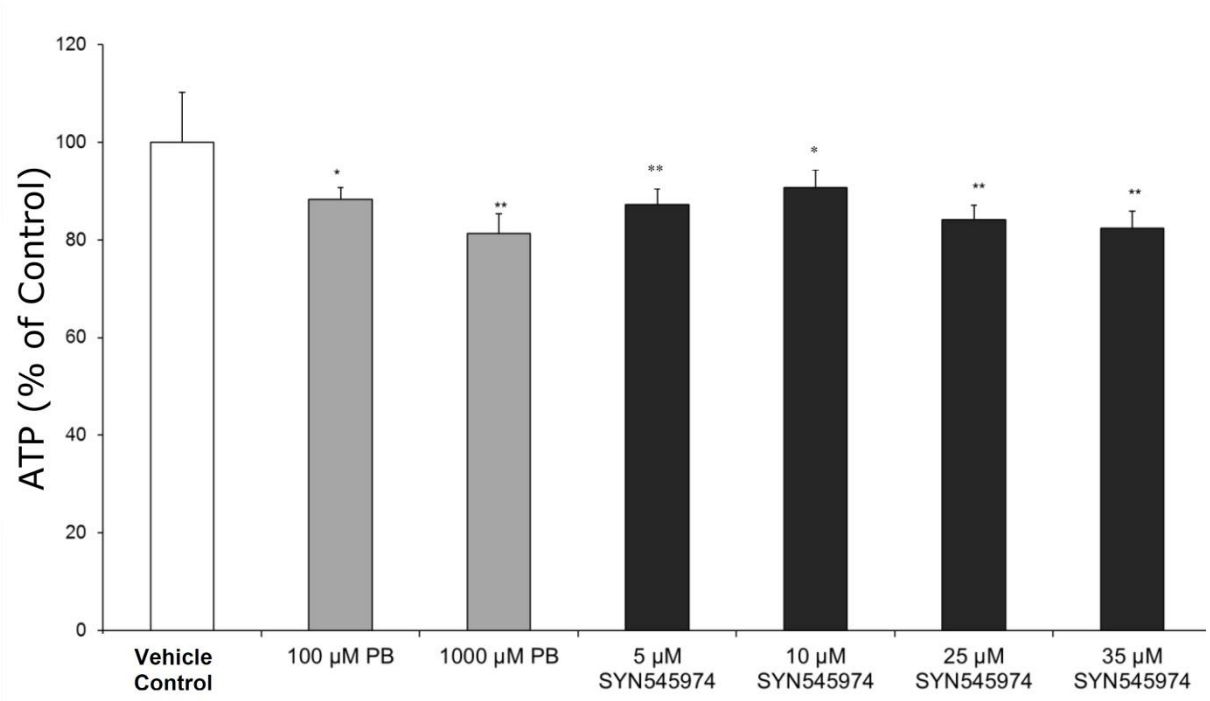
- Activation of the constitutive androstane receptor (CAR).
- An early, transient, increase in hepatocellular proliferation.
- Increased hepatocellular foci as a result of clonal expansion of spontaneously mutated (initiated) cells.
- Eventual progression to form liver tumours.

And the following associative events:

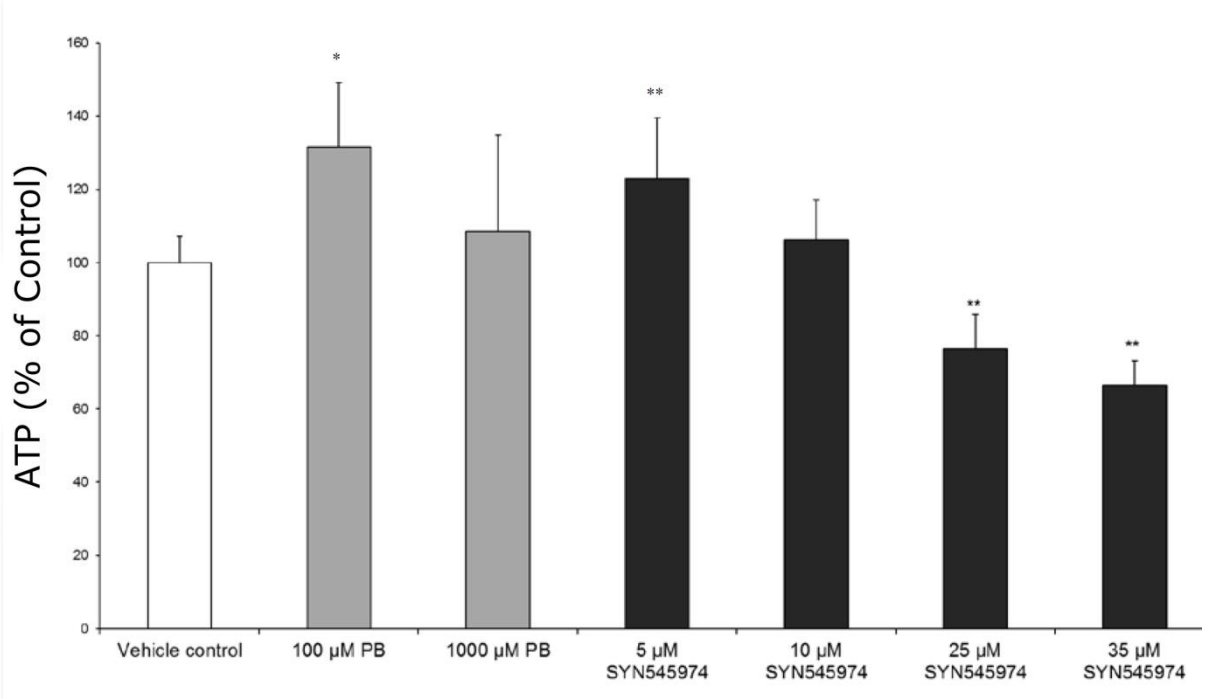
- Increased expression of genes encoding cytochrome P450s (CYPs), particularly Cyp2b/Cyp3a isoforms.
- Increased incidence of hepatocellular hypertrophy.
- Increased liver weight.

While no transgenic knockout animal or cell models nor mRNA induction studies were used in the investigation into the MoA, and this is a valid criticism of the pydiflumetofen data package, experimental data demonstrated that pydiflumetofen did not produce the key event of cell proliferation in human liver cells *in vitro*. However, it must be noted that the tests in *in-vitro*

human hepatocytes were conducted with only one donor which limits the degree of certainty as to whether this effect matches the known species differences that have been demonstrated for other CAR activators. Also noted is the greater sensitivity of the human hepatocytes to cytotoxicity with pydiflumetofen treatment (figure below). Hepatocyte toxicity was assessed following 96 hours of culture and was indicated by ATP depletion. Significant cytotoxicity (as measured by decreases in ATP) was observed at levels > 10 uM such that meaningful results for replicative DNA synthesis with higher concentrations of pydiflumetofen are lacking. In mouse hepatocytes there was also cytotoxicity but only at higher levels of pydiflumetofen as illustrated by a significant depletion of ATP (≥ 56.8% of control) and therefore cytotoxicity with concentrations ≥ 50 µM (preliminary study CXR1490). Human hepatocytes are more sensitive to pydiflumetofen toxicity. Based on such limited test samples it is difficult to conclude on a qualitative difference in the established CAR activation MoA for hepatocarcinogenesis between rodents and humans.



(A) Mouse hepatocytes. ATP content as mean % of control ± standard deviation, (n=6). A one-way ANOVA followed by the Dunnett test was performed on the results; * statistically different from control $p<0.05$; ** $p<0.01$



(B) Human hepatocytes. ATP content as mean % of control ± standard deviation, (n=6 replicates, 1 donor liver). A one-way ANOVA followed by the Dunnett test was performed on the results; * statistically different from control $p<0.05$; ** $p<0.01$.

Figure: *In Vitro* Hepatocyte cytotoxicity – ATP levels. (A) Pooled mouse hepatocytes. (B) Liver hepatocytes from a single human donor.

It must be further noted that data to investigate alternative modes of action is limited. It has not been adequately demonstrated with pydiflumetofen that other mechanisms are not also at work. There is some increase in EROD and LAH enzyme expression but no mRNA induction studies were performed to investigate alternative or CAR-supporting pathways. No positive controls for aryl-hydrocarbon receptor (AhR) activation/CYP1A induction (e.g. 3-methylcholanthrene, omeprazole) or LAH expression/CYP4A induction (e.g. fibrate drugs, other peroxisome proliferators) were investigated so it is not possible to put into context the small increases in EROD and LAH that were observed. Other effects of CAR activation such as the suppression of apoptosis were also not investigated, though it is recognised that some crosstalk with AhR (and/or CYP1A induction) may be indicative of apoptotic suppression. Consequently the data package supplied in support of the primary MoA is not as robust as some of those supplied in the past for other substances presented for consideration by RAC.

An explanation for the differential sensitivity between male and female mice with respect to the development of liver tumours is also lacking. The tumours were only observed in male mice. In the *ex-vivo* enzyme analysis of liver samples study (table below, #5) PROD activity in females is 50-80% reduced relative to males at day-7 and day-28, respectively, implying an attenuated CAR response in female mice; this however remains speculative.

6.3.2 The proposed Mode of Action for pydiflumetofen induced liver tumours

Activation of CAR in male mice results in altered expression of CAR-responsive genes leading to CAR-mediated stimulation of cell proliferation (and associated replicative DNA synthesis, figure below). This promotes an environment which enables increased cell replication to occur, which can result in a higher rate of spontaneous mutations due to normal replication errors. Combined with suppression of apoptosis, this promotes an environment that would allow a spontaneously mutated cell to clonally expand before it could be removed by normal apoptotic control processes.

Over time, transformed cells progress to pre-neoplastic foci, with clonal expansion eventually leading to the development of liver tumours. The activation of CAR and a subsequent burst of cellular proliferation are considered to be key events in the tumour MoA, being necessary and directly resulting in the induction of liver tumours in the mouse.

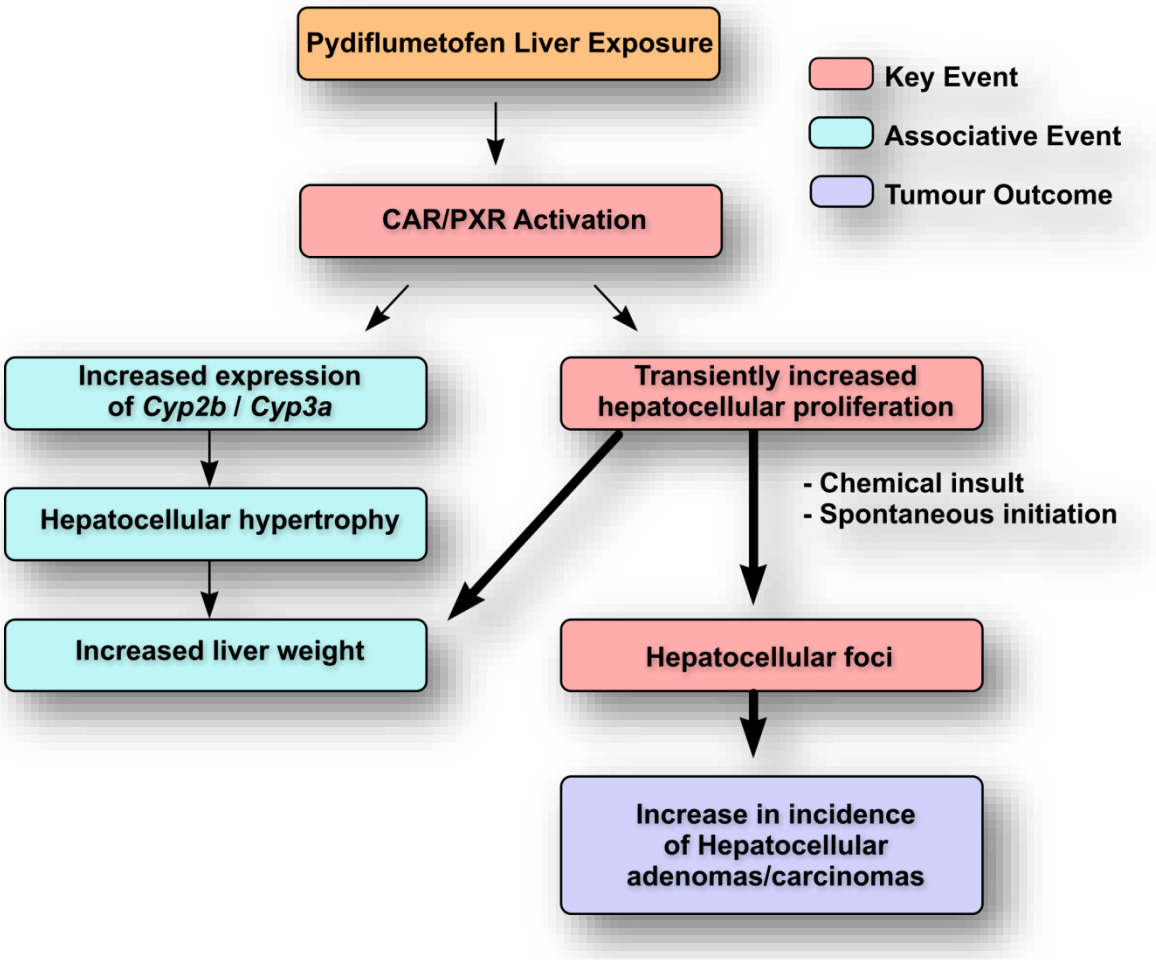


Figure: Mode of Action hypothesis for pydiflumetofen-induced liver tumour formation in male mice.

The effects on cytochrome P450s are considered to be associative events in that while they are a characteristic hallmark of CAR activation, they are not central to the induction of liver tumours. A further associative event is liver hepatocellular hypertrophy, which is caused by proliferation of the smooth endoplasmic reticulum as a consequence of cytochrome P450 induction. This hypertrophy, in combination with the increased hepatocyte proliferation, in turn results in an increase in liver weight.

The mechanistic studies showed the following results (see summaries in the table below):

1. Pydiflumetofen increased mouse and human hepatocyte activity of Phase I xenobiotic metabolising enzymes consistent with activation of CAR/PXR nuclear receptors.
2. Pydiflumetofen was a direct activator of CAR from the mouse, rat and human, and was efficacious in all three species.
3. Pydiflumetofen did not increase hepatic peroxisomal β-oxidation (PCO) but did have a limited increase in LAH (surrogate for CYP4A).
4. Liver weight increased with concomitant hepatocellular hypertrophy.

5. Pydiflumetofen increased replicative DNA synthesis in a PB-like manner in mouse hepatocytes. However the increase was not that convincing and a clear dose response relationship is not evident (part A of the figure below)
6. Pydiflumetofen increased PROD activity in preference to BQ by about 5-fold *in vivo* in the mouse liver.
7. Pydiflumetofen did not increase replicative DNA synthesis in human hepatocytes although the study has been conducted with only one donor.
8. Pydiflumetofen had a small effect on EROD activity.

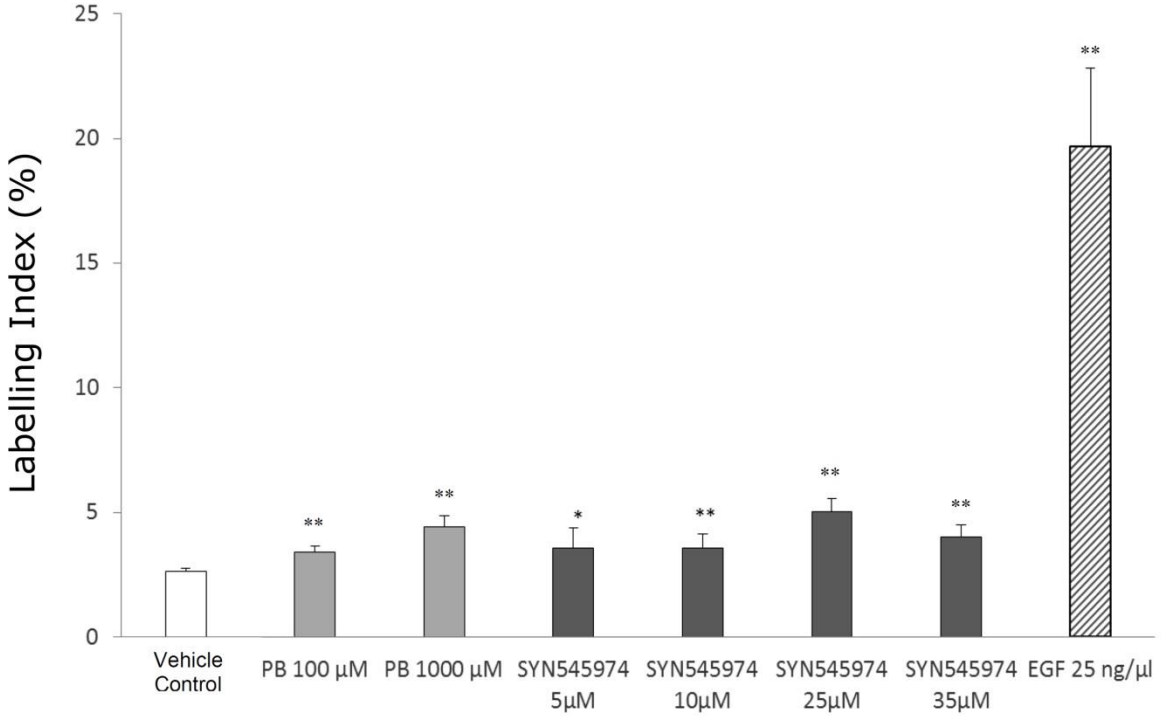
Table: *RAC Summary of mode of action studies investigating liver tumours*

Endpoints investigated	Summary observations	Reference
1. *In-vitro* CAR3 Transactivation Assay with Mouse, Rat and Human CAR - CAR 3 activation explored via cDNA expression vectors transfected into monkey COS-1 cells, along with necessary cofactors and a CYP2B6 response element-luciferase reporter construct. - Table 40 CLH report - Pydiflumetofen clearly interacts with and activates the CAR receptor construct from 3 species: the rat, mouse and human in a positive dose response manner. The data indicate that it is a generic CAR activator similar to Artemisinin (typically used as a reliable positive control for CAR activation in *in vitro* studies).	1. Pydiflumetofen is a direct human and rodent CAR activator. 2. Selective species positive controls robustly activated the appropriate CAR. **30 µM** rat CAR3 ↑ 42 fold mouse CAR3 ↑ 20 fold human CAR3 ↑ 15 fold **10 µM** rat CAR3 ↑ 37 fold mouse CAR3 ↑ 32 fold human CAR3 ↑ 13 fold **3 µM** rat CAR3 ↑ 14 fold mouse CAR3 ↑ 34 fold human CAR3 ↑ 5 fold **1 µM** rat CAR3 ↑ 2.8 fold mouse CAR3 ↑ 24 fold human CAR3 ↑ 1.5 fold	Omiecinski, (2014)
2. *In-vivo* 28-day mouse oral (dietary) study - CD-1 strain - Males only. - Evaluate liver effects - Treatment: 2, 7, and 28 days - Pydiflumetofen tested (0, 10, 324 mg/kg/day) - Total cytochrome P450 levels - Enzymes: PROD only. - Strong positive dose response for PROD activity - Brd immunostaining - Table 40 CLH report	**a. Liver:** 1. ↑ Abs and Rel wt at high dose 2. ↑ Centrilobular hepatic hypertrophy 3. ↑ Mitotic cells in liver after 2 days **b. Enzyme activity:** 1. Pydiflumetofen induced substantial PROD activity at all timepoints indicative of CAR activation. **c. Replicative DNA Synthesis:** 1. ↑ BrdU incorporation with initial high burst at day 2, lower thereafter. **2250 ppm (324 mg/kg/day)** ↑ absolute liver weight 22% and 24% after 28 and 7 days respectively ↑ liver:body weight ratio 28% and 21% after 28 and 7 days respectively ↓ 40% AST activity 28 days ↑ cytochrome P450 approx. 2-fold after 2, 7 and 28 days	Anonymous (2015)

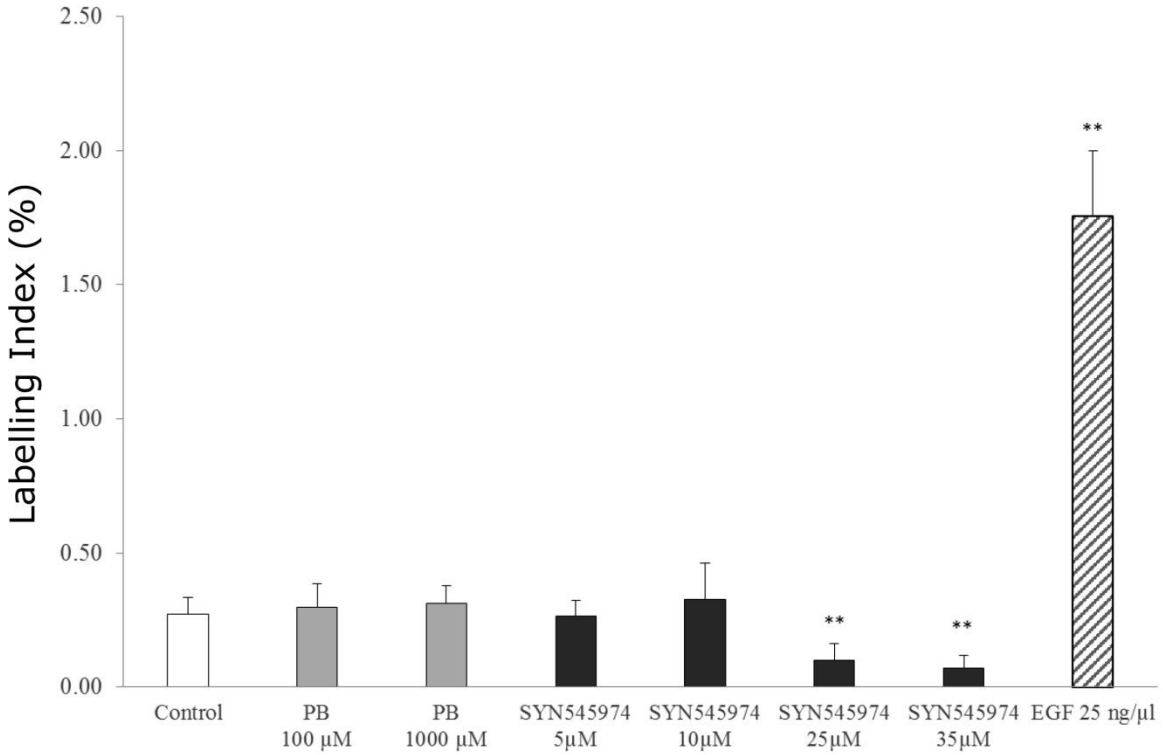
	↑ PROD activity 28, 36 and 37-fold after 2, 7 and 28 days respectively (marker of Cyp2b activity) ↑ BrdU incorporation 14-, 5 and 6-fold after 2, 7 and 28 days respectively ↑ Centrilobular hepatic hypertrophy in 9/10, 9/10 and 10/10 animals after 2, 7 and 28 days respectively ↑ Mitotic cells in liver 7/10 after 2 days **75 ppm (10 mg/kg/day)** ↑ PROD activity 1.9. 1.6 and 2.4-fold after 2, 7 and 28 days respectively not statistically significant ↑ BrdU incorporation 3.1, 2.3- and 5.6-fold after 2, 7 and 28 days respectively	
3. *In-vitro* mouse hepatocytes - CD-1 strain - Cells from male animals - Pydiflumetofen, PB, EGF tested - low [pydiflumetofen] tested (5, 10, 25 and 35 µM) - Table 40 CLH report	**a. Cytotoxicity:** 1. ↓ ATP by 9-20% No dose effect up to 35 µM pydiflumetofen. **b. Enzyme induction:** 1. Pydiflumetofen induces PROD and BROD activity indicative of CAR and PXR activation. In absolute terms, mouse expression levels are higher than in human hepatocytes, typically > 200-fold. **c. Replicative DNA Synthesis:** 1. Pydiflumetofen increases labelling index in a PB-like manner, dependent on CAR activation, positive EGF control. The proliferative signal is much stronger in mouse hepatocytes relative to human hepatocytes, typically by about 10-fold. **All groups** ↓ 9.3-17.6% ATP as an indicator of cytotoxicity **35 µM** ↑ 50.6% hepatocyte proliferation as measured by replicative DNA synthesis ↓ PROD and BROD enzyme activities (not statistically significant), possibly indicating substrate competition or signs of cytotoxicity **25 µM** ↑ 89.8% hepatocyte proliferation as measured by replicative DNA synthesis ↓ PROD and BROD enzyme activities (not statistically significant) **10 µM** ↑ 71.3 and 97.0% respectively PROD and BROD enzyme activities **5 µM** ↑ 84.6 and 98.5% respectively PROD and BROD enzyme activities	Lowes (2015a)
4. *In-vitro* human hepatocytes - 1 male donor. - Pydiflumetofen, PB, EGF tested - low [pydiflumetofen] tested (5, 10, 25 and 35 µM) - Table 40 CLH report	**a. Cytotoxicity:** 1. ↓ ATP by 34% (i.e. 67% relative to control values) at 35 µM pydiflumetofen and ↓ ATP by 24% at 25 µM → significant cytotoxicity. **b. Enzyme induction:** 1. Pydiflumetofen induces PROD and BROD activity indicative of CAR and PXR activation. **c. Replicative DNA Synthesis:** 1. No effect on cell proliferation (S-phase of the cell cycle). Positive EGF control. **35 µM** ↓ 34 % ATP as an indicator of cytotoxicity ↑ 164 and 218% respectively PROD and BROD enzyme activities **25 µM** ↓ 24 % ATP as an indicator of cytotoxicity	Lowes (2015b)

	↑ 155 and 326% respectively PROD and BROD enzyme activities **10 µM** ↑ 236 and 491% respectively PROD and BROD enzyme activities **5 µM** ↑ 191 and 237% respectively PROD and BROD enzyme activities	
5. *Ex-vivo* Enzyme Analysis of Liver Samples - CD-1 mouse livers. - Treatment: 3, 7, and 28 days - Dietary study - [pydiflumetofen] 0, 500, 1500, 4000 and 7000 ppm - Table 40 CLH report	Alternate modes of action were only investigated via enzyme activity; there were no mRNA expression studies performed. No increase in peroxisome palmitoyl CoA oxidation and only minimal increase (<4fold) in microsomal LAH, indicating pydiflumetofen (SYN545974) is not a peroxisome proliferator. EROD (marker for Cyp1a activity), no biologically significant effects. Dose related increase in hepatic total P450 content to maximum of 1.8 and 1.6 fold (males and females) at 7000 ppm on day 28**.** **a. 28-day treatment:** 1. PCO: ↓ 0.7-0.5x (M-F) peroxisomal B-oxidation 2. total cytochrome P450: ↑ up to 2x 3. EROD: ↑ 1.7-1.4x (M-F) 4. PROD: ↑ 15-3x (M-F) 5. BQ: ↑ 3.5-2.5x (M-F) 6. LAH: ↑ 3.5-1x (M-F) **b. 7-day treatment:** 1. PCO: ↓ 0.7-0.4x (M-F) peroxisomal B-oxidation 2. total cytochrome P450: ↑ up to 2x 3. EROD: ↑ 2-1.3x (M-F) 4. PROD: ↑ 21-11x (M-F) 5. BQ: ↑ 5.4-2.5x (M-F) 6. LAH: ↑ 2-0.7x (M-F) **c. 3-day treatment:** 1. PCO: ↓ 0.7-0.8x (M-F) peroxisomal B-oxidation 2. total cytochrome P450: ↑ up to 2x 3. EROD: ↑ 3.6-2x (M-F) 4. PROD: ↑ 10-13x (M-F) 5. BQ: ↑ 4.4-3.5x (M-F) 6. LAH: ↑1.8-1.3x (M-F)	Anonymous (2012)

The available experimental data for pydiflumetofen indicate that the CAR-mediated MoA is the most plausible mechanism for induction of the mouse liver tumours. Mechanistic studies indicate a direct activation of CAR from different species (the rat, mouse and human) by pydiflumetofen. Results also demonstrated that the associative events of the proposed CAR mediated mechanism, i.e. induction of enzymes specific to CAR/PXR occur in human hepatocytes (but at a very low level relative to mouse enzyme induction). However, the key event, proliferation, (essential for subsequent tumour formation, figure below) is not observed in primary human hepatocytes at levels of pydiflumetofen of 5 and 10 µM. It is plausible from the available data that the liver carcinogenicity in mice proceeds via CAR activation, which is a tumour mechanism with little relevance to humans, however, significant uncertainties in the key study are a cause of concern and the level of increase in DNA replicative synthesis in mouse cells does not seem to follow a convincing dose response relationship. Indeed at the highest dose tested (35 µM) there is a drop in DNA replicative synthesis (part A of the figure below).



(A) Mouse hepatocyte replicative DNA synthesis - labelling index as mean ± standard deviation (n=5). A one-way ANOVA followed by the Dunnett test was performed on the results; * statistically different from control $p<0.05$; ** $p<0.01$.



(B) Human hepatocyte replicative DNA synthesis - labeling index as mean ± standard deviation (n=5). A one-way ANOVA followed by the Dunnett test was performed on the results; ** statistically different from control $p<0.01$. Concentrations of pydiflumetofen > 10 µM were cytotoxic and not informative on DNA replicative potential.

Figure: Mechanistic data - *In Vitro* Hepatocyte S-Phase labelling. (A) Pooled mouse hepatocytes. (B) Liver hepatocytes from a single human donor.

The *in vitro* hepatocyte studies (*Lowes 2015a, b*) with mice and human hepatocytes are key studies in the assessment of the MoA. The lack of proliferation in human hepatocytes seems to be considered as the ultimate proof for the proposed MoA. However, at the concentrations where proliferation of mice hepatocytes was shown (25 and 35 µM), there seems to be cytotoxicity in the human hepatocytes making the interpretation of the data rather difficult. Thus, following exposure of the human cells to 25 and 35 µM, the ATP-concentration decreased substantially (24-34% relative to controls) meaning that at least one quarter to one third of the human cells had died, thus it is doubtful if it would have been possible to detect potential proliferation. The data might indicate a rather large difference in sensitivity between rat and human hepatocytes to pydiflumetofen toxicity or that the human hepatocytes were in a worse condition than the mice hepatocytes, making them more vulnerable to toxic insult.

Details about the human donor (a human hepatocyte quality certificate was supplied in the original study report), indicated that the liver was from a middle aged (51yr) and healthy caucasian male with no history of tobacco, alcohol or drug abuse and with negative serology for viruses such as HIV-1 & 2; HTLV 1 & 2; CMV, HBV, and HCV.

Cytotoxicity was apparent with the human hepatocytes, since concentrations of pydiflumetofen >10 µM showed significant decreases in ATP relative to concurrent controls. In mouse hepatocytes there was significant depletion of ATP content (≥56.8% of control) following the administration of pydiflumetofen at concentrations of 50 µM and higher (CXR1490; preliminary study). It appears that human hepatocytes are more sensitive to pydiflumetofen toxicity. Note, pydiflumetofen belongs to the class of succinate dehydrogenase inhibitors or SDHI fungicides, a mitochondrial complex II inhibitor and therefore is designed to inhibit oxidative phosphorylation and ATP production in target fungi.

There is no evidence to suggest that the human hepatocytes were in a worse condition than the mouse hepatocytes thereby rendering them more vulnerable to toxic insult. From the graphs in the figure above, it can be seen that they tolerated high doses of phenobarbital and 5 and 10 µM pydiflumetofen as well if not better than the mouse hepatocytes. There was some historical control data supplied with respect to the performance of human hepatocytes but it is not well documented. It is thought that the historical control data referred to the same hepatocyte donor source, i.e. donor 8210 as used in the human hepatocyte *in vitro* study. The S-phase basal labelling index was similar but cells from donor 8210 had lower responses to PB and EGF relative to the HCD and induced levels of PROD and BROD enzyme activity were also lower than HCD (table below).

Table: *In Vitro Hepatocyte Proliferation Index and Enzyme Activity Measurements in Male Human Hepatocyte Cultures used to investigate pydiflumetofen.*

Test Item	**Lowes (2015)**	**HCD (alternate study, same donor?)**
S-phase labelling index: Vehicle control PB (1000/500) EGF, 25 ng/ml	 0.27 ± 0.06 0.31 ± 0.06 / 1.76 ± 0.24	 0.33 ± 0.09 / 0.41 ± 0.09 4.50 ± 0.89
PROD activity (pmol resorufin/min/mg): Vehicle control PB (1000/500)	 0.11 ± 0.017 0.37 ± 0.045	 0.191 ± 0.022 0.463 ± 0.019
BROD activity: (pmol resorufin/min/mg) Vehicle control PB (1000/500)	 1.15 ± 0.06 8.53 ± 0.65	 1.49 ± 0.20 8.158 ± 0.441

Basal levels or responses may be a way to assess hepatocyte health. The table above illustrates some variability in response by human hepatocytes from the same donor, but without access to a more substantial database from several human donors it is difficult to assess the functional quality of the hepatocytes from the *Lowes* (2015b) study. For instance, the reduced response to 25 ng/mL EGF (1.76 vs 4.5); perhaps this could indicate a reduction in the health of the donor hepatocytes or just reflect the natural variation expected amongst hepatocytes to xenobiotics in their environment.

The data from the *in vitro* S-phase labelling study illustrated that the hepatocytes were capable of responding to a positive control proliferative stimulus (figure above). Typically the EGF proliferative response is weaker in human hepatocytes relative to rodent hepatocytes by about 10-fold. This has been seen for other *in vitro* human hepatocyte proiferative assays investigating CAR agonists (e.g. silthiofam). The human hepatocytes appear to be sufficiently viable and able to tolerate high levels of phenobarbital and pydiflumetofen up to 10 µM. Higher levels of pydiflumatofen are clearly cytotoxic to the human hepatocytes so that the only reliable controls to indicate proliferative capacity and non-proliferative capacity lie with the positive control EGF and the negative control (in humans) with phenobarbital.

The main limitation with the *in vitro* human hepatocyte study is the reliance on cells from a single donor.

6.4 Dose selection

Dose selection is considered to be an issue. While the MTD was technically achieved, female rats could have been dosed higher than 102 mg/kg/day, certainly at a level at least equivalent to that of the males (≈ 300 mg/kg/day). Extensive explanations have been provided by the applicant and RMS in the DAR.

Dose levels were selected after evaluation of the rat subchronic (90 day) and pharmacokinetic studies with pydiflumetofen. These studies demonstrated a clear non-linear exposure in males and females and were indicative of a saturation of absorption as the dose increased. Statistical analysis to assess the proportional relationship between pharmacokinetic parameters and dose demonstrated an exposure/dose-proportional relationship between 5-300 mg/kg bw/day for males and 5-100 mg/kg bw/day in female rats.

The top dose level therefore was established where kinetic proportionality was lost due to dose-limited absorption of pydiflumetofen. Higher oral doses resulted in less and less compound being absorbed (per increment in dose) into the blood. However, this argument for the dosing regimen employed in studies may be regarded as insufficient because there is no plateau achieved in the increase in absorption. Systemic exposure continues to increase with doses higher than the highest dose levels selected for the long-term and reproductive toxicity studies. Comparison between plasma AUCs determined after administration of the phenyl radiolabelled active substance in rat at dose levels up to 1000 mg/kg/day, showed that systemic exposure still increased beyond 100 or 300 mg/kg/day:

i. by 4-fold between 100 - 1000 mg/kg bw/d

ii. by 1.7-fold between 300 - 1000 mg/kg bw/d

Further support for increased systemic exposure at very high doses was confirmed in the short-term repeated dose studies where an increase in toxicity (liver and body weight effects) was observed with increasing dose. A higher dose administered to female rats in the long-term study

for instance, would have confirmed whether the increase in the thyroid follicular adenomas was a real substance related effect or not.

RAC agrees with the DS that technically the long-term rodent studies satisfy the MTD requirement. The DS did not explain the rationale behind the dose selection. They only noted the DAR RMS concern on whether the doses were sufficient to account for the presence of the primary metabolite 2,4,6 trichlorophenol (2,4,6-TCP). RAC considers all scientific approaches to dose selection as long as all the inherent hazards of the substance can be evaluated with confidence. The pharmacokinetic approach in this case did not provide the necessary confidence for concluding fully on toxicity and therefore also on classification. Top dose selection in the long-term and reproductive toxicity studies is not considered sufficient. RAC notes the concerns of the RMS on the dosing regimens employed and considers that a dose beyond the linear range of absorption should have been tested because it was apparent that systemic exposure continued to increase with increasing dose.

6.5 Conclusions

6.5.1. Human Relevance

1. There is uncertainty regarding a possible treatment-related increase in thyroid adenoma /hyperplasia observed in female rats. The mechanistic investigation in males was inappropriate (since this was the less sensitive sex for thyroid effects) and the top dose for females was too low to adequately assess the tumourigenic response in this organ.
2. The available data shows that pydiflumetofen activates human CAR and induces Cyp2b/3a-related enzyme activity, indicating that CAR binding and activation is also relevant for humans. However, the central tenet that CAR-mediated hepatocarcinogenesis in rats and mice is of no relevance to liver tumour promotion in humans is based on the supposition that CAR-mediated gene activation in humans produces only a subset of responses observed in mice and rats. While CAR activation can occur in humans as it does in mice and rats, there is so far no evidence that human CAR regulates genes involved in cell growth leading to replicative DNA synthesis and liver hyperplasia.
3. It has not been adequately demonstrated in the case of pydiflumetofen that there are qualitative differences between humans and rodents, particularly in the ultimate key event of cell proliferation. The limited *in vitro* study with human hepatocytes showed significant cytotoxicity at concentrations > 10 µM so that no firm conclusions regarding cell proliferation can be made. Cells from only one donor were used and the results cannot be interpreted as being representative from a cross section of the human population.
4. It has not been adequately demonstrated with pydiflumetofen that other mechanisms are not also at work. There is some increase in EROD and LAH enzyme expression but no mRNA induction studies were performed to investigate alternate or CAR-supporting pathways. No positive controls for aryl-hydrocarbon receptor (AhR) activation/CYP1A induction (e.g. 3-methylcholanthrene, omeprazole) or LAH expression/CYP4A induction (e.g. fibrate drugs, other peroxisome proliferators) were investigated so it is not possible to put into context the small increases in EROD and LAH that were observed. Other effects of CAR activation such as the suppression of apoptosis were also not investigated though it is recognised that some crosstalk with AhR (and/or CYP1A induction) may be indicative of apoptotic suppression.

5. It has not been adequately demonstrated with pydiflumetofen that the CAR activation model was primarily responsible for the neoplastic response in mouse liver. No transgenic knockout animals or humanised receptor models were employed to provide further support for CAR-mediated liver tumours of little to no relevance to humans.

6.5.2 Conclusion on Carcinogenicity

1. The mechanistic data that are available indicate a mechanism-of-action (MoA) via CAR activation as the most plausible mechanism responsible for the liver tumours in male mice. The tumour profile observed in the pydiflumetofen carcinogenicity bioassay was typical of a non-genotoxic mechanism (single species, single sex and single organ involvement without decreased latency).
2. The data from the rat studies inadequately informs on thyroid carcinogenicity potential due to insufficient dosing and limited mechanistic investigation. The available thyroid data in the rat are not considered to be sufficient for classification. In female rats the incidence of benign follicular cell adenoma (3/51) and follicular cell hyperplasia (3/51) at the top dose of 102 mg/kg bw/day was at the upper boundary of the HCD range for that tumour. Some data suggests that it could be related to an increase in hepatic-mediated thyroxine clearance. Mechanistic studies were not performed using the more sensitive female animals. The maximum test dose to female rats in the rat carcinogenicity study was too low.
3. The data from the mouse study shows a strong liver carcinogenicity potential for pydiflumetofen in males. Several mechanistic studies were performed to show support for a CAR-mediated MoA for the mouse liver tumours. The concern for tumours arising from this MoA is only reduced if it is adequately demonstrated that (1) alternative MoAs are eliminated and (2) there are qualitative differences between humans and rodents, particularly regarding the ultimate key event, cell proliferation. It is the view of RAC that these two points have not been adequately addressed with the pydiflumetofen data. Therefore, even though there is reason to believe that a CAR-mediated mechanism of liver tumour promotion seems very plausible, the weight of evidence is weak and RAC cannot conclude on there being no hazard to humans. Classification with Carc 2 is proposed.

6.5.3. Classification into category 1A

There is no information from studies in humans to inform on carcinogenic potential and so classification in category 1A is not supported.

6.5.4 Classification into category 1B

The substance was not found to be genotoxic. Tumours were restricted to one organ (liver), to one species (mouse) and one sex (males), there was no evidence for a reduction in liver tumour latency. There was a progression to malignancy and there was an apparent dose response relationship. The incidence of thyroid adenomas (female rats) was within the historical control range. Overall the data was considered to show limited evidence of a carcinogenic effect and not sufficient to warrant classification in category 1B.

6.5.5. Classification into category 2

The data supports a category 2 classification for pydiflumetofen. The main weakness in the evidence base is that hepatocytes from only one donor were used to demonstrate that pydiflumetofen lacks proliferative ability in this tissue in humans. On the weight of the presented

evidence, a CAR mode of action was considered to be the most plausible explanation for the increase in liver adenomas and carcinomas in the male mouse. However, the shortcomings in the hepatocyte studies, in combination with the other uncertainties (such as selection of doses in both rats and mice, possible treatment-relationship of thyroid adenoma/hyperplasia observed in female rats, absence of MoA data for female rats, possible involvement of carcinogenic metabolite 2,4,6-TCP) need to be considered. A weight of evidence assessment by RAC considered that it was not conclusively shown that the tumours were of no relevance to humans. RAC concludes there is sufficient uncertainty to **warrant classification as Carc. 2** for pydiflumetofen.

6.5.4 No Classification

Overall RAC considers insufficient evidence has been presented to indicate no concern for human health; there is insufficient data to conclude on other alternative modes of action; and that whether the sole MoA for liver tumours in mice were secondary to hepatocellular proliferation induced by activation of the CAR/PXR nuclear receptors has not been adequately addressed.

RAC evaluation of reproductive toxicity

Summary of the Dossier Submitter's proposal

Pydiflumetofen was evaluated for reproductive and developmental toxicity in a multi-generation reproductive toxicity study in the rat (*Anon., 2015*) and in pre-natal developmental toxicity studies in the rat (Preliminary study; *Anon, 2011* and Main study; *Anon., 2015*) and rabbit (Preliminary study; *Anon., 2015a* and Main study; *Anon., 2015b*). All studies were guideline (OECD 416 and OECD 414) and GLP compliant.

1. Sexual function and fertility

In a two-generation reproduction toxicity study, four groups of 24 male and 24 female rats of the Crl:WI(Han) strain were administered pydiflumetofen orally *via* the diet at 0, 150, 750 or 4500 ppm (males) or 0, 150, 450 or 1500 ppm (females) from 10 weeks before pairing and until necropsy.

No treatment-related deaths or clinical signs occurred in either the P or F_1 generation parents or litters. Body weight gain was slightly (but stat. sig.) reduced in P and F_1 generation males at 4500 ppm during the initial weeks of the pre-pairing periods, with slight effects on food intake only seen during the F_1 generation. There was no effect of treatment on female body weight gain at any concentration, in either generation.

Relative liver weights were increased in males at 750 and 4500 ppm in both generations, in the P generation females at 450 ppm and in the P and F_1 generation females at 1500 ppm. Microscopic findings in the liver (diffuse hepatocyte hypertrophy) were only seen at 4500 ppm in P and F_1 generation males and 1500 ppm in P generation females only. Microscopic findings in the thyroid (minimal follicular epithelial hypertrophy) were seen in males of both generations given 4500 ppm.

A slight increase in oestrous cycling at 1500 ppm in the P generation females was within the HCD range and was not observed in the F_1 females; fertility and mating performance or gestation length were not affected for either generation at any dietary concentration. All pregnant females gave birth to live litters with a similar number of pups born, and there was no effect of treatment on the postnatal survival of P or F_1 generation litters to Day 21 of age.

Sexual maturation was slightly delayed for F_1 generation males given 4500 ppm and females given 1500 ppm which was considered by the DS to be possibly related to treatment. The delay was associated with reduced body weight gain in males but not in females. However, this was considered to be of questionable relevance as there was no effect on related parameters such as oestrus cycling, mating performance or fertility and no effect on ano-genital distance of F1 generation pups. Sperm parameters for males from either generation or on the number of the F1 generation ovarian follicles were unaffected.

In parental males the LOAEL for systemic toxicity was 4500 ppm (P/F_1 - 276.6/363.8 mg/kg/day, respectively), based on a reduction in body weight gain during the early pre-pairing period and increased liver and thyroid weights associated with microscopic changes (diffuse hepatocyte hypertrophy and thyroid follicular epithelial hypertrophy). In females, the LOAEL was 1500 ppm (P/F_1 - 116.2/140.6 mg/kg bw), based on increased liver weight associated with hepatocyte hypertrophy.

The reproductive NOAEL was considered to be in excess of 4500 ppm for P and F_1 generation males (276.6 and 363.8 mg/kg/day, respectively) and 1500 ppm for females in the P (116.2 mg/kg/day (pre-pairing)) and F_1 generations (140.6 mg/kg/day (pre-pairing)).

The NOAEL for offspring toxicity was considered to be in excess of 4500 ppm for P and F_1 generation males (276.6 and 363.8 mg/kg/day, respectively) and 450 ppm for female in the P (36.1 mg/kg/day (pre-pairing)) and F_1 generations (42.4 mg/kg/day (pre-pairing)) based on the delayed sexual maturation observed at 1500ppm.

It was the conclusion of the DS/RMS that there were no effects to warrant classification of pydiflumetofen for fertility. In addition, the DS/RMS was of the opinion that the findings (skeletal variants and sexual maturation) were not sufficiently convincing to be a basis for classification of pydiflumetofen as a developmental toxicant.

2. Development

Developmental toxicity was investigated in the rat and the rabbit in GLP and guideline compliant studies with preliminary range-finding studies for both.

2.1 Rat studies

A preliminary developmental toxicity study was conducted in the rat at doses of 0, 100, 200, 500 and 1000 mg/kg/day. The only treatment related effect was a transient reduction in bodyweight gain at 500 mg/kg/day and a slight body weight loss for females given 1000 mg/kg/day on day 6 to 7 of gestation. No maternal or developmental toxicity was seen at the highest dose tested.

For the main developmental toxicity study in the rat, dose levels of 0, 10, 30 and 100 mg/kg/day were evaluated (*Anon., 2015*). An initial reduction in body weight and food intake in response to the onset of dosing with 100 mg/kg/day on day 6 was resolved by day 9 with no overall adverse effects on either parameter. There was no evidence of developmental toxicity at any dose level. The incidences and intergroup distribution of major, minor and variant foetal abnormalities were considered not to be related to administration of pydiflumetofen. Based on the results of this study, the NOAEL for maternal toxicity is considered to be 30 mg/kg/day and the NOAEL for embryo-foetal development is considered to be 100 mg/kg/day.

In the 2-generation study in rats (*Anon., 2015*), sexual maturation was delayed in F_1 generation males given 4500 ppm and in females given 1500 ppm. In males, the delay in sexual maturation was considered by the DS to be secondary to reduced body weight gain and not a direct effect of treatment with pydiflumetofen. In females, the delay was not secondary to reduced body weight gain. In a conservative approach, this finding was considered sufficient to establish the

NOAEL (offspring) of the study at 36 mg/kg/day but was not considered to be of sufficient toxicological significance to be relevant for classification because:

i. There was no subsequent effect on related parameters such as oestrus cycling, mating performance or fertility observed in the F1 generation pups.

ii. There was no effect on ano-genital distance of F1 generation pups

No effect on endocrine or reproductive organs were observed in all the available repeated toxicity studies database (rat, mice, dog)

2.2 Rabbit studies

A preliminary developmental toxicity study was conducted in the rabbit at doses of 0, 250, 500 and 1000 mg/kg/day. The maternal NOAEL was 500 mg/kg/day based on decrease in maternal body weight gain at 1000 mg/kg/day. No developmental toxicity was seen at the highest dose level.

For the prenatal developmental toxicity study in the rabbit, dose levels of 0, 10, 100 and 500 mg/kg/day were evaluated (*Anon., 2015c*). No maternal effects were observed in the study. A marginally increased incidence of rib cartilage variant (one or more costal cartilage interrupted) was observed at 100 and 500 mg/kg/day without a clear dose response relationship but above the HCD from the conducting laboratory. In a conservative approach, the NOAEL for embryo-foetal development was considered to be 10 mg/kg/day.

In conclusion, the RMS was of the opinion that these findings in rats (skeletal variants and sexual maturation) are not sufficiently convincing to be a basis for classification of pydiflumetofen as a developmental toxicant.

Comments received during public consultation

Industry (comment 7)

This comment referred to the observation of a statistically significantly increased foetal (but not litter) incidence of a rib variant 'one or more costal cartilage interrupted' at 100 and 500 mg/kg bw/day in the main rabbit developmental toxicity study (*Anon., 2015c*).

Historical control data were submitted by the industry (*Manton, J., 2018*) which refers specifically to the background occurrence of the rib variant 'one or more costal cartilage interrupted'. In the table below the incidence in 54 studies from the relevant supplier is described from 2007 to 2017.

Table. *Variant skeletal findings with statistical significance*

Observations	**Dose levels (mg/kg bw/day)**			
	0 (control)	**10**	**100**	**500**
Skeletal examination				
No. of foetuses (F)	*163*	*142*	*132*	*154*
No. litters (L)	*22*	*18*	*19*	*21*
Rib: one or more: costal cartilage interrupted (variant)	F: 8 (4.4%) L: 6 (27.3%)	F: 8 (5%) L: 6 (33.3%)	F: 14 (14%) L: 12 (63%)*	F: 12 (8%) L: 10 (47.6%)*
HC data (Manton, J., 2018)	**54 studies (2007-2017):** **Foetal range 4-13 (2.6% - 9.4%)** **Litter range 4-9 (25% -42.8%)**			

The individual study data indicated that this variation occurred in 8 of 54 studies; between 2.6 and 9.4% of foetuses were affected in 4 – 9 litters. It was not clear whether the remaining studies had either zero incidence of the variant or it was not recorded or not looked for.

After the public consultation, industry submitted three position papers addressing the adequacy of dose selection, the liver carcinogenic response in male mice and the delay in sexual maturation in rats in the 2-generation study.

The position paper on the delay in sexual maturation was entitled - Company "Comments: Pydiflumetofen - Explanation of Developmental Delays in Rat Multigeneration Study". They concluded:

1. "*Reductions in body weight during post-natal development are known to cause delays in the onset of puberty. It is established in the scientific literature that growth rate is of greater importance than arrival at a particular fixed weight in determining the onset of puberty. For both the high dose F1 female and male pups there is clear evidence of a delay in growth during the post-natal period, and this can explain any apparent difference in the time of sexual maturation in these groups.*
2. *Further evidence that administration of pydiflumetofen does not directly perturb sexual maturation comes from the F2 generation which were examined on PND 0 for differences in anogenital distance (AGD). There was no difference in AGD in the F2 generation at any dose level. Furthermore, no other endpoint on the study, which could indicate abnormal progression through puberty, was different from the control group (e.g. there was no effect on oestrus cyclicity in the females, and no effect on time to mating or overall mating performance).*
3. *The individual animal data do not show the treated groups, including the highest doses tested, to fall outside of a normal physiological range.*
4. *Taking the above together there are compelling reasons to consider that any apparent specific changes in sexual maturation are unrelated to pydiflumetofen*".

MSCA (Comment 8)

A Member State considered that the following malformations/variations in the rat and rabbit developmental toxicity studies may be treatment-related and relevant for classification.

1. Cleft palate/palatine and/or malformed palate at 100 mg/kg bw/day in the rat main study and at 1000 mg/kg bw/day in the rabbit range-finding study.
2. rib variants (costal cartilage) in rat at 100 mg/kg bw/day and rabbit at 100 and 500 mg/kg bw/day.

They also argued that the test substance was insufficiently tested as minimal toxicity was seen at the highest dose tested in the rat which was within the linear kinetic phase of the absorption rate. Likewise it was noted that in the rabbit the AUC was only slightly increased with increased oral dosing (AUC increase by only 1.2 and 4.2 fold following a dose increase of 10 (dose of 100 mg/kg/day) and 50 fold (dose of 500 mg/kg/day), respectively) while a single incidence of malformed palate was seen in litters of dams tested to 250 and 1000 mg/kg bw in the preliminary study.

Assessment and comparison with the classification criteria

3. Assessment of reproductive and developmental studies

3.1 Fertility

3.1.1 Introduction

The potential reproductive toxicity of pydiflumetofen was investigated in a GLP and Guideline compliant study up to dietary levels of 4500 ppm (P/F_1; 276.6/363.8 mg/kg bw) in males and 1500 ppm (P/F_1 ; 116.2 and 140.6 mg/kg/bw) in females.

3.1.2 Dose selection

In this study, minimal systemic toxicity was evident at any dose level, with no clinical signs or mortalities. This, it could be argued, may be a deviation from OECD guidelines where (according to OECD 416) dose selection criteria specify "*..the highest dose level should be chosen with the aim to induce toxicity but not death or severe suffering*". Minimal transient effects on weight gain (males), increased relative liver weight with diffuse hepatocyte hypertrophy at doses ≥ 750 ppm were seen in males and at 450 ppm in females. Minimal thyroid follicular cell hypertrophy was seen (males). The toxicokinetic profile of pydiflumetofen following repeated gavage or dietary dosing in rats was determined and used to support dose level selection for studies including the 2-gen study based on linear *versus* non-linear kinetics in rat.

It was argued in the DAR (Vol 3CA B6.1 (iii) p.17) that the dose selection based on TK data is only applicable to the parent as the additional studies were performed using a non-radiolabelled method which does not permit following the fate of the metabolites. The active substance is extensively metabolised, hence circulating levels of parent are extremely low compared to the metabolites, especially 2,4,6 TCP and its sulphated conjugate. The applicant considered that the non-proportionality of pydiflumetofen kinetics with increasing dose (due to dose limited absorption) would be reflected by non-proportionality in the formation of all metabolites. However, the non-proportionality of pydiflumetofen kinetics means that systemic exposure (measured by AUC(0-t)) stops increasing linearly with the dose, but it doesn't mean that systemic exposure does not continue to increase at all with higher doses than the maximal dose employed for the reproductive toxicity studies (100-300 mg/kg/day (female-male) in rat studies.

This is confirmed both by the available toxicokinetic and toxicity studies performed. Table 6.1-3 in the DAR (reproduced below) presents a comparison between the plasma AUCs measured after oral administration in rat of phenyl radiolabeled pydiflumetofen (permitting a follow-up of the active substance and its phenyl metabolites including 2,4,6 TCP) at the dose levels of either 5, 100 (female), 300 (male) or 1000 mg/kg bw/d. These toxicokinetic data showed that systemic exposure still increases beyond 100 or 300 mg/kg bw/day in rat: by 4-fold between 100 mg/kg bw/day and 1000 mg/kg bw/d and by 1,7-fold between 300 mg/kg bw/d and 1000 mg/kg bw/d.

Table: *Comparison of AUC_{0-t} for Total Radioactivity in Plasma Following a single Oral Administration of [phenyl-U-14C]- pydiflumetofen to Male and Female Rats*

Dose (mg/kg)	**Fold increase**		**PLASMA**	
			$AUC_{(0-t)}$(*ng*	**Fold increase**
5		Male	8830	
		Female	10200	
5		Male	8705	
		Female	7890	
100	20	Female	113000	14
300	60	Male	316000	36
1000	200	Male	546000	62
		Female	450000	44

Therefore, the maximal doses used in the multi-generation study were not optimal and this raises the issue of whether the endpoints were fully investigated or indeed if the study was truly OECD 416 guideline compliant with regard to the selection criteria for determining the highest dose.

3.1.3 Parental effects

Effects of treatment at the higher doses consisted of ;

1. reduced body weight gain in P and F_1 generation males given 4500 ppm during the initial weeks of pre-pairing, with slight effects on food intake only in the F_1 generation. There was no effect of treatment on female body weight gain at any dietary concentration, in either generation.
2. no effects on fertility and mating performance or gestation length for either generation at any dietary concentration. Sperm parameters were unaffected. All pregnant females gave birth to live litters with a similar number of pups born, and there was no effect of treatment on the postnatal survival of P or F_1 generation litters to Day 21 of age.
3. oestrous cycling; high dose females had slightly longer and fewer oestrous cycles during the 21 days before the pairing period ($p<0.05$). However, cycling parameters for all females were within the normal range (DAR Table 6.6.1-6). This effect may have been driven by 2 females with longer mean cycle length (5 and 4.5 days) and was not reproduced in the F_1 generation. Therefore, any differences are biologically insignificant and unrelated to treatment.
4. An increase in liver and thyroid weights was associated with microscopic changes (diffuse hepatocyte hypertrophy and thyroid follicular epithelial hypertrophy) for both the P and F_1 generations (see the highlighted in the table below).

***Table**. P Generation Oestrous cycle length and periodicity*

Observation		**Dose Group (ppm)**			
		0	**150**	**450**	**1500**
No. of oestrous cycles (over 21 days)	Mean	4.8	4.7	4.6	4.5
	SD	0.4	0.5	0.5	0.5
	N	24	24	24	24
Oestrous cycle length (days)	Mean	3.93	3.95	3.95	4.05*
	SD	0.10	0.13	0.11	0.24
	N	24	24	24	24

***Table**. Intergroup comparison of selected organ weights in P animals*

		Dietary Concentration (ppm)							
		Males				**Females**			
		0	**250**	**750**	**4500**	**0**	**150**	**450**	**1500**
Liver	Absolute	13.10	13.62	14.23 *(+9%)*	**17.19**** *(+31%)*	13.35	13.44	13.96 *(+5%)*	**15.28**** *(+14%)*
	Adjusted	12.96	13.12	**14.18**** *(+9%)*	**17.88**** *(+38%)*	13.24	13.38	**14.05*** *(+6%)*	**15.36**** *(+16%)*
	Relative‡	3.09	3.14	3.37 *(+9%)*	4.27 *(+38%)*	4.89	4.94	5.20 *(+6%)*	5.67 *(+16%)*
Kidney	Absolute	2.63	2.62	2.62	2.72	2.07	2.08	2.10	2.14
	Ajusted	2.61	2.55	2.61	**2.82**** *(+8%)*	2.05	2.07	2.12	2.15
	Relative‡	0.62	0.61	0.62	0.68	0.76	0.76	0.78	0.80
Thyroid	Absolute	0.022	0.024	0.024	**0.025*** *(+14%)*	0.017	0.016	0.017	**0.015*** *(-12%)*
	Adjusted	0.022	0.023	0.024	**0.026**** *(+18%)*	0.017	0.016	0.017	**0.015*** *(-12%)*
	Relative‡	0.005	0.005	0.006	0.006	0.006	0.006	0.006	0.006

*statistically significant p<0.05
**statistically significant p<0.0

Table 20. *Intergroup comparison of selected organ weights in F[1] animals*

		Dietary Concentration (ppm)							
		Males				Females			
		0	250	750	4500	0	150	450	1500
Liver	Absolute	13.06	13.39	13.93	**16.47**** *(+26%)*	11.30	11.09	12.01	**13.24**** *(+17%)*
	Adjusted	12.43	12.82	**13.93**** *(+12%)*	**17.63**** *(+42%)*	11.26	11.09	12.05	**13.23**** *(+17%)*
	Relative‡	3.02	3.10	3.35 *(+11%)*	4.27 *(+41%)*	4.47	4.43	4.83	5.30 *(+18%)*
Kidney	Absolute	2.59	2.54	2.56	2.60	2.12	2.06	2.09	2.15
	Ajusted	2.52	2.47	2.56	**2.74**** *(+9%)*	2.11	2.06	2.10	2.15
	Relative‡	0.60	0.59	0.62	0.68	0.84	0.82	0.84	0.86
Thyroid	Absolute	0.020	0.022	0.022	**0.023*** *(+15%)*	0.019	0.020	0.019	0.019
	Adjusted	0.019	**0.022*** *(+16%)*	**0.022*** *(+16%)*	**0.024**** *(+26%)*	0.018	0.020	0.019	0.019
	Relative‡	0.005	0.005	0.005	0.006	0.007	0.008	0.008	0.008

*statistically significant p<0.05
**statistically significant p<0.01

3.1.4 Offspring effects

There were no deaths or clinical signs considered related to treatment in either F_1 or F_2 pups. Anogenital distance was not affected in either generation.

All litter and viability parameters were unaffected by treatment. Mean body weight from day 7 and mean weight gains at 1500 ppm were slightly (but stat. sig.) lower over Days 1 – 21 in the F_1 generation litters only (table below).

Table 21. *F_1 and F_2 intergroup comparison of bodyweights/body weight gains*

Lactation Day	Dose Group (ppm)							
	0	150	450	1500	0	150	450	1500
	F_1 Pups - male & female				F_2 Pups - male & female			
1	6.69	6.64	6.72	6.45	6.23	6.45	6.60	6.35
4a	10.17	9.98	10.30	9.27	8.16	7.81	8.76	8.05
7	16.83	15.99	16.50	**14.89****	12.60	12.32	13.47	12.95
14	33.97	32.75	33.11	**29.91****	30.32	29.58	30.93	29.33
21	51.63	50.63	50.28	**46.29****	48.94	47.27	49.60	47.32
D1-21	44.95	43.96	43.57	**39.84****	42.44	40.70	42.93	40.69

a - Before standardisation (culling)
** - Statistically different from control, p<0.01

3.1.4.1 Sexual maturation F_1 Pups

Exposure to pydiflumetofen delayed the age of onset of preputial separation in males and vaginal opening in females (table below). Both effects were statistically significant but only the preputial separation was outside the historical control range for males at the highest dose. There is no

evidence to determine if the delays have been caused by direct effects on the genital tract or by effects on systemic endocrine function.

Rat 2-gen study – Points to note:

- *In Males:* Sexual maturation was delayed (stat. sig.) at 4500 ppm (45.9 days versus 43.0 days in controls). This was considered by the DS to be related to lower body weight in males given 4500 ppm indicating that the delay in sexual maturation of males was secondary to reduced body weight gain during the lactation period. However, the decrease in body weight was not dose-related amongst the treated groups and varied between 9-12% lower than controls in the high dose group. The association of the reduced preputial separation with reduced weight may be considered equivocal in this case.
- *In Females:* Sexual maturation was also delayed (stat. sig. $p<0.01$) at 1500 ppm (30.3 and 33.0 days for control and 1500 ppm females, respectively). There was no association with reduced body weight gain in female pups during the lactation period.
- There is no evidence to conclude that the delay in sexual maturation is secondary to a reduction in the rate of body weight development and not a direct effect of pydiflumetofen. Prior experience with other substances considered by RAC (e.g. fluxapyroxad) where there were very clear and more pronounced reductions in the rate of post-natal body weight development across two-generations showed little to no effect on pubertal milestones, i.e. no delay in time to BPS in males or vaginal opening in females. On this basis time-to-puberty endpoints in the context of post-natal body weight changes need to be assessed carefully.
- The rat 2-gen study does not fully inform on these endpoints, given that the high dose selected for females was too low. Pubertal data was only available for one generation, the P-generation offspring (F1 juveniles). There were no data available for the F1-generation offspring (F2 juveniles) for puberty endpoints because the pups were sacrificed on PND-21.

Table. *Sexual maturation and body weights (g); F_1 pups only.*

Males						
Observation			**Dose Group (ppm)**			
			0	**150**	**750**	**4500**
Preputial Separation	Day of age	Mean	43.0	43.2	44.1	45.9**
		SD	2.4	2.7	2.3	3.5
		N	24	24	24	24
	Body weight	Mean	191.9	189.8	193.3	183.9
		SD	18.3	16.3	15.5	12.7
		N	24	24	24	24
Females						
Observation			**Dose Group (ppm)**			
			0	**150**	**450**	**1500**
Vaginal Opening	Day of age	Mean	30.3	31.3	31.8	33.0**
		SD	2.1	2.6	2.1	2.5
		N	24	24	24	24
	Body weight	Mean	97.2	100.3	103.7	105.7
		SD	11.1	16.5	12.4	11.8
		N	24	24	24	24

** - Statistically different from control, $p<0.01$

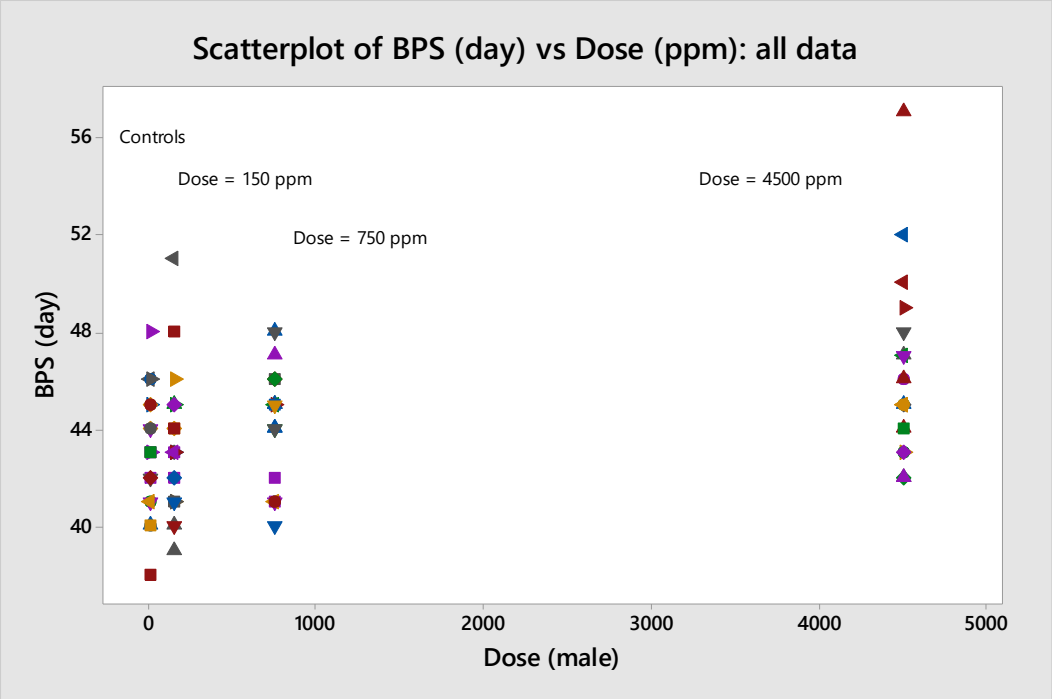
Offspring toxicity

Offspring toxicity		
Males	750 ppm (59 mg/kg/day)	No effects
	4500 ppm (364 mg/kg/day)	F1: delayed sexual maturation (45.9 days versus 43.0 days in controls)
Females	450 ppm (42.4 mg/kg/day)	No effects
	1500 ppm (116 mg/kg/day)	F1: delayed sexual maturation (33.0 days versus 30.3 days in controls). No subsequent effect on oestrus cycling, mating performance or fertility and no effect on ano-genital distance

The vaginal opening was delayed by around 3 days and the delay was statistically significant at 1500 ppm ($p<0.01$), although within the range of the historical control data submitted. The age of vaginal opening is dependent on both body weight at weaning and body weight on the day of vaginal opening (*Edwards and Kay, 1985*). There was no effect on related parameters such as oestrous cycling (in the F_1 generation), mating performance or fertility, and there was no effect on ano-genital distance of F_1 generation pups. However, as weight gain was not affected, the delay in vaginal opening may be due to the delay in development during the lactation period and a relationship to treatment also cannot be excluded (see DAR Table 6.6.1-21). Moreover, the delay is more than 2 days which should be considered as treatment related and generally adverse, unless it is seen as a delay in general growth. RAC noted that effects were seen in both sexes, outside the HCD range (for males) and cannot be explained on the basis of bodyweight change alone, therefore the effects were considered to be clearly treatment related. Reproductive organ weights were unaffected by treatment and there were no abnormalities found at necropsy which were related to treatment.

3.1.4.2 Detailed examination of Pubertal Data

Simple scatterplots (figure below) of the pubertal raw data are not very informative with respect to comparing dose groups with the controls.



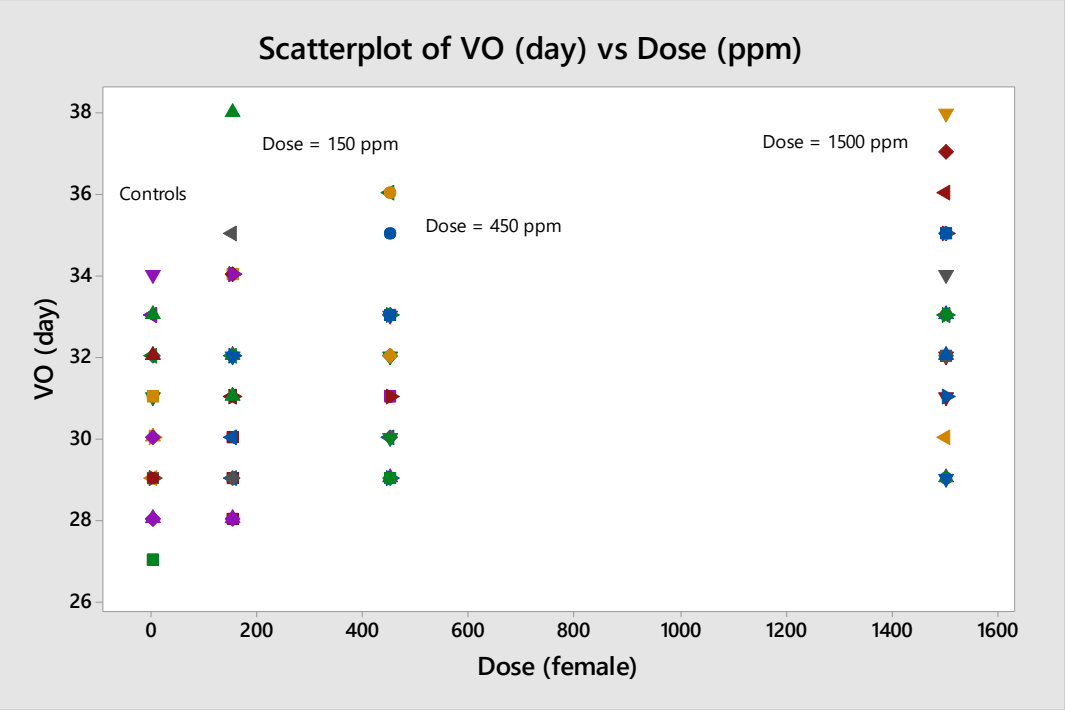
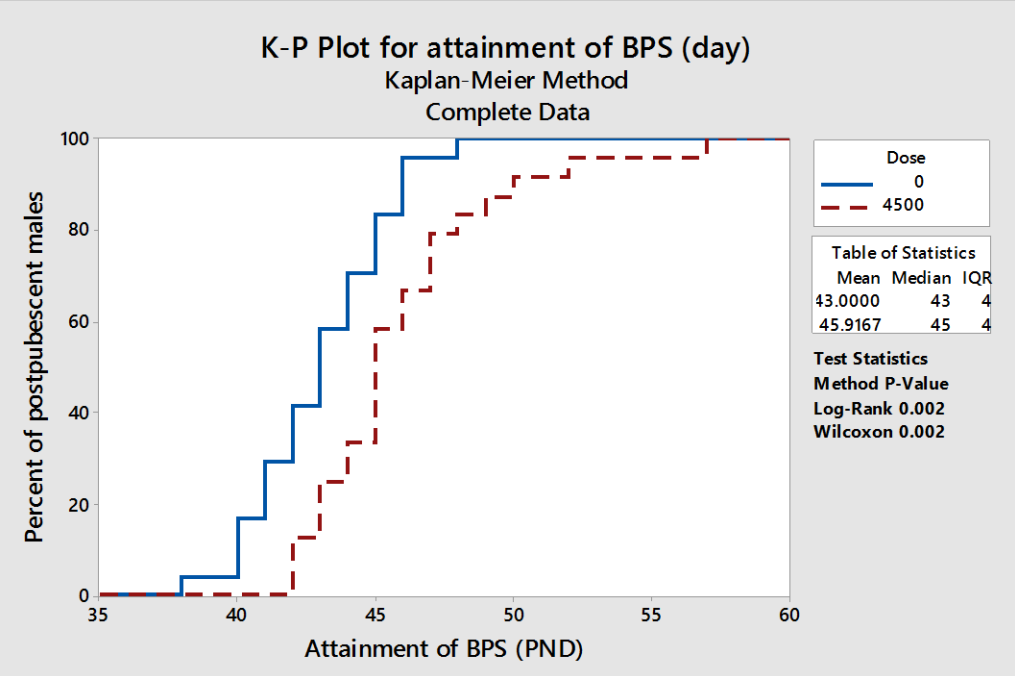


Figure: simple scatterplots of all individual pubertal day of attainment data.

A more convenient way to compare all the data within a dose group to that of the controls is to generate Kaplan-Meier Curves for data with differing times-to-event endpoints. Pubertal data is ideal to compare in this way because the measured endpoint is a fixed, timed event, i.e. post natal day of BPS in males, post natal day of vaginal opening in females. In this case the comparative analysis depends upon the whole "curve" and is not unduly disrupted by isolated data points (figure below).



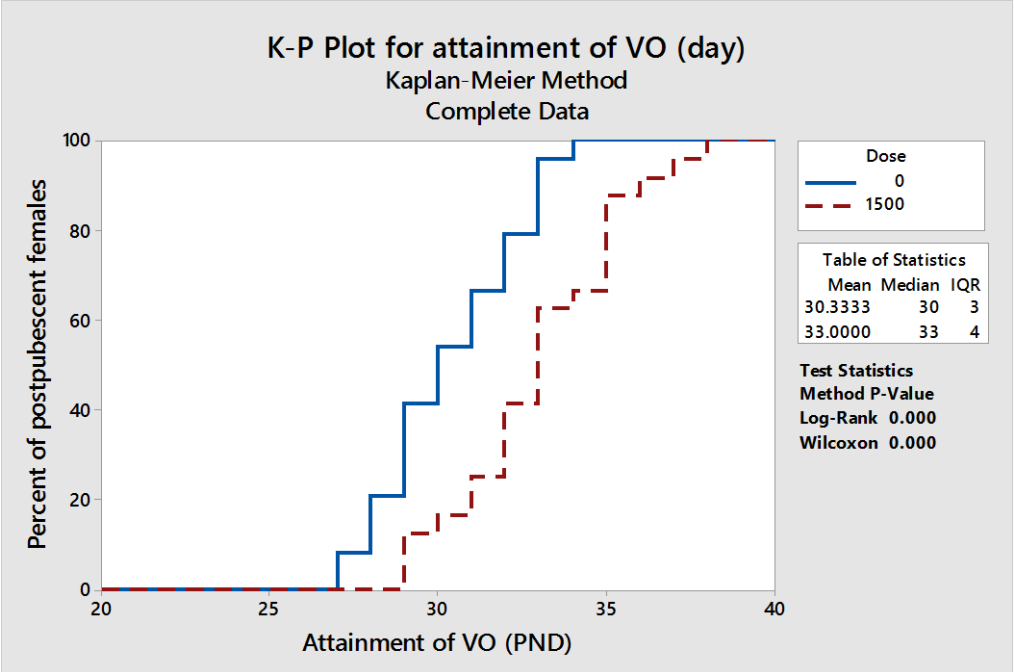


Figure: Kaplan-Meier Curves for Pubertal data endpoints.

Seqani historical control data for balanopreputial reparation and vaginal opening

The mean value at 4500 ppm for the postnatal day of balanopreputial separation sits outside of the Seqani mean HCD range (2008-2014: 43.0 – 45.3 days). This is driven largely by 1 of the 24 males in this group, which is not representative of the remaining 23 treated males and background control animals. Exclusion of this male as an outlier from the 4500 ppm dose group gives a mean value of 45.4 days, which is at the upper boundary of the HCD range. The use of Kaplan-Meier plots, however, allows for the comparison of all data points and still shows a significant delay in BPS associated with the high dose group animals. If the HCD are confined to within +/- 5 years of the 2-gen rat study, then the Seqani (see the table below) HCD (3 studies) for mean attainment of BPS becomes 43.0 – 43.5 days, illustrating that the PND of BPS in the

high dose group may be even more significant because it now falls well outside the upper boundary of the HCD.

Summary of Sequani historical control data (six studies: 2008 – 2014)

The raw data was not available, only summary data from the original study report, for the rat 2-gen study (2015).

HCD from performing lab

Year	BPS	BW	VO	BW
Jan. 2008	45.2	184.8	32.7	92.3
Mar. 2009	45.0	177.8	34.1	98.1
Jan. 2009	45.3	193.4	31.8	89.3
Jan. 2014	43.0	172.3	31.3	88.1
Feb. 2014	43.5	183.4	29.3	89.4
Feb. 2014	43.3	179.6	29.9	85.7
mean	44.2	181.9	31.5	90.5
sd	1.0	6.5	1.6	3.9
range (all years):	38-52		25-39	
range (2014):	38-47		25-35	

3 studies within the relevant date range (+/- 5 years)

3 older studies had greater delays in BPS and VO

Other sources of historical control data

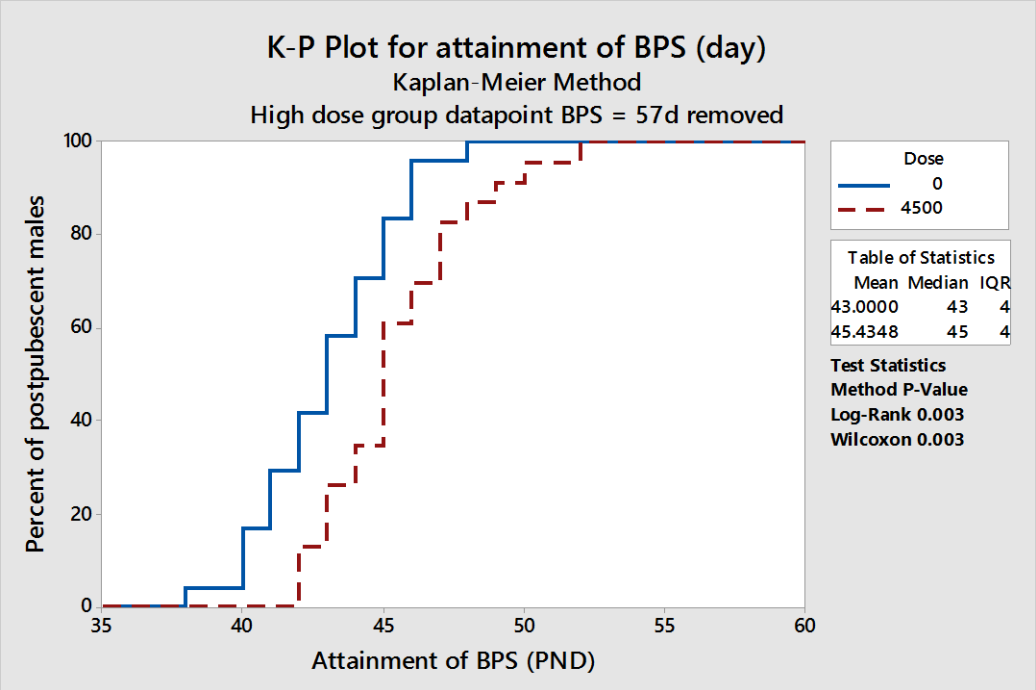
Sexual maturation data for Wistar rats was also available from the 2018 report "Reproductive Toxicology Historical Control Data in Rats" published by Charles River laboratories. Data from six studies was available but no date for the studies was provided.

Males: BPS: mean = 44.7 days; range 43.3 – 45.9 days, n = 87 animals

Females: VO: mean = 31.8 days; 30.7 – 32.5 days, n = 85 animals.

Revised analysis of BPS in the high dose group:

Exclusion of one male as an outlier from the 4500 ppm group gives a mean value of 45.4 days, which is at the upper boundary of the HCD range. The use of Kaplan-Meier plots (figure below) enables the comparison of all data points and still shows a significant delay in BPS associated with the high dose group animals.



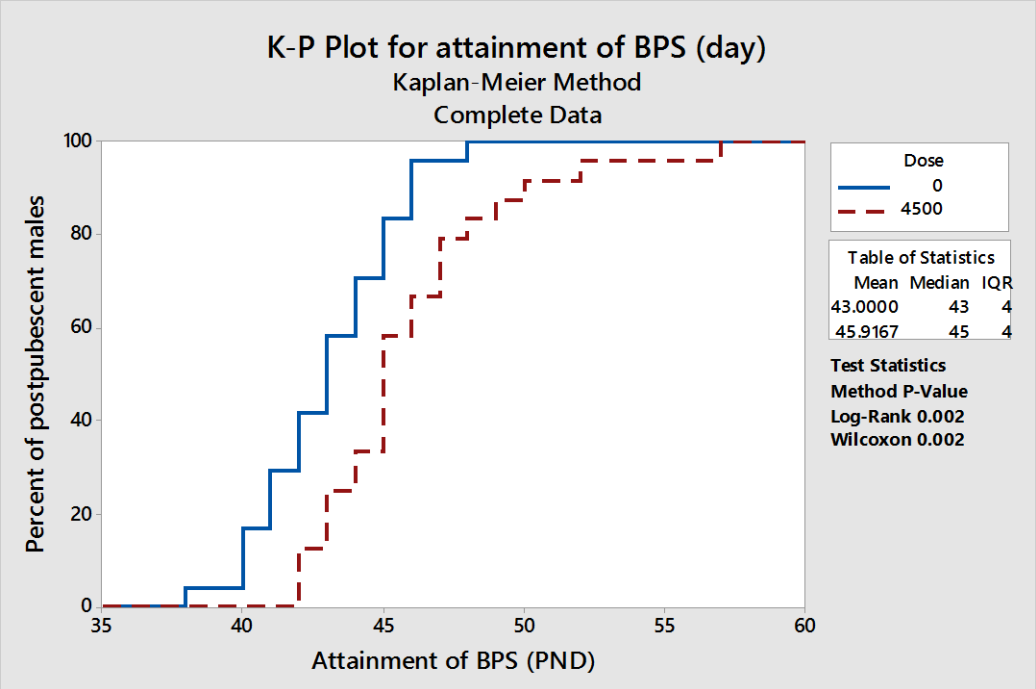


Figure: Kaplan-Meier Curves for adjusted BPS and original BPS pubertal data.

3.1.4.3 Comparison with the Criteria for Classification

Under CLP (annex I: 3.7.1.3) it is recognised that adverse effects on sexual function and fertility include effects on the onset of puberty. This criterion is satisfied for pydiflumetofen, since it delays the time of onset for balanopreputial separation (BPS) in males and vaginal opening in females. The classification criteria as specified under section 3.7.2 of CLP indicate classification as Repr. 2 is appropriate when there is some evidence from animal studies "*..of an adverse effect on sexual function and fertility,..*"

Published literature generally shows that delays in pubertal endpoints by substances due to endocrine-mediated mechanisms occur together with numerous other effects. For example,

known anti-androgens responsible for significant delays in BPS in males include flutamide, prochloraz, and vinclozolin but the effects are not solely confined to one specific event but occur together with other evidence that may include changes in nipple retention, anogenital distance/anogenital index and sex organ weights, as well as gross and histopathological findings. In female rats, atrazine, propazine and esfenvalerate prolong or delay vaginal opening by a number of days, often through centrally acting mechanisms that perturb the hypothalamic-pituitary control responsible for puberty attainment. These other effects are not apparent in rats treated with pydiflumetofen. The fact that both male and female pubertal endpoints are delayed may indicate a more general central acting mechanism or general toxicity. There is no mechanistic data however to explain the delayed attainment of the pubertal endpoints.

Reductions in body weight during post-natal development are known to cause delays in the onset of puberty. It is established in the scientific literature that growth rate is of greater importance than arrival at a particular fixed weight in determining the onset of puberty. For both the high dose F1 female and male pups there is evidence of a delay in growth amounting to a 9-12% reduction relative to concurrent controls during the initial (PND 1-21) post-natal period. However, much larger effects seen for another substance (fluxapyroxad) previously considered by RAC across two generations did not show these effects on pubertal age endpoints, therefore it is recognised that growth rate data must be interpreted with care.

The key question is whether the effects on pubertal attainment alone are sufficient to justify classification for such a significant adverse hazard to human reproduction. The weight of the available evidence suggests an absence of such a hazard (no alteration on the timing of the first dioestrus and of oestrus or mating performance or fertility have been reported; no effect on ano-genital distance of F1 generation pups; no effect on endocrine or reproductive organs were observed in any of the available repeated toxicity studies; no effect on follicle counts in the ovaries).

3.1.4.4 Increased pup death in the F1 generation litters (F2 pups)

Table 9.2 in the original study report summarises F1 generation litters (F2 pups). Curiously the [Missing (presumed cannibalised)] and [Found dead/killed prematurely] entries show very high numbers of lost pups when compared with the data for the F0 generation litters (F1 pups, Table 9.1 of the study report) (see the highlighted in the tables below). Also, in the summary of pregnancy and litter data (for the F1 generation parents) it was stated that 4, 4, 1 and 5 females in the groups given 0, 150, 450 or 1500 ppm, respectively, showed total litter loss between days 2 and 9 of age. Since these litter losses were spread across the groups, including controls, and showed no dose response relationship, they were considered not to be treatment related. However, no explanation has been provided for this effect and hence they do raise questions regarding the quality of the original study.

3.1.5 Conclusion

Dietary administration of pydiflumetofen up to 4500 ppm for males and 1500 ppm for females for two successive generations, was well tolerated. There was a slight reduction in early body weight gain of males at 4500 ppm of both the P and F_1 generations. Microscopic changes were seen in the liver (diffuse hepatocyte hypertrophy) and thyroid (follicular epithelial hypertrophy) of high dose P and F_1 generation males; the liver changes were also seen for high dose P generation females and generally correlated with organ weight changes.

RAC agrees with the DS that the maximal dose level tested should have been higher than the linear kinetics dose range although effects on liver and thyroid weight and histology were apparent at the higher doses in males and less so in females.

There were no adverse effects on reproductive performance, mating behaviour or conception. A statistically significant delay in vaginal opening without reduced body weight was observed in females given 1500 ppm which may be associated with a delay in development during the lactation period or other undefined effects. A statistically significant delay in preputial separation in males at 4500 ppm may have been associated with a slight but not statistically significant reduction in body weight, but again other factors may be involved. RAC noted that effects are seen in both sexes, at the upper boundary of the historical control data (for males) and these cannot be explained by bodyweight changes alone, therefore an association with treatment cannot be excluded.

3.2 Development

3.2.1 Rat

3.2.1.1 Preliminary study

A preliminary study investigated the effects of the test item on the pregnant rat and on embryonic and foetal development (Days 6 to 19 of gestation), in order to select dose levels for a subsequent developmental toxicity study.

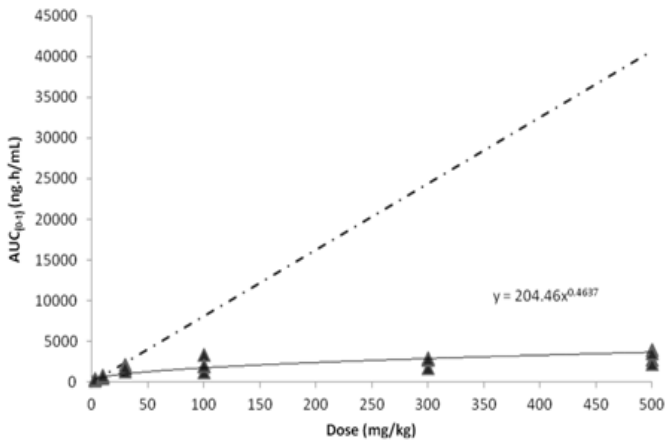
Oral administration to 6 females per group of 0, 100, 200, 500 and 1000 mg/kg bw/day was well tolerated. Reduction in body weight gain for females given 500 mg/kg/day and body weight loss for females given 1000 mg/kg/day, were observed between Days 6 to 7 of gestation as the only maternal findings.

There was no effect of treatment on the mean number of corpora lutea, mean number of implantations, the extent of pre- or post-implantation losses or on the mean number of live foetuses. All pregnant females had litters with live foetuses at scheduled necropsy. There was no effect of treatment on mean foetal weights, mean litter weights or the percentage of male foetuses. Mean placental and gravid uterus weights were similar across the groups.

Relevant findings

Significant effects on weight gain from 500 mg/kg bw/day. On the basis of this data, a higher dose should have been selected in the main study to properly assess the hazard according to the OECD guideline, even though above 100 mg/kg/d dosage, the total systemic increase becomes non-linear with an increase by 1.3 fold when the dose increases by 3 (from 100 to 300 mg/kg/d) and by 1.5 fold when the dose increases by 5 (from 100 to 500 mg/kg/d).

Relationship between AUC(0-t) and dose in female rats following daily oral administration of pydiflumetofen for 7 days (Figure 6.1.1-3 of the DAR):



3.2.1.2 Main study

Time-mated female Crl:CD (SD) rats were dosed by oral gavage from Day 6-19 of gestation within the vehicle, 1 % carboxymethylcellulose, at dose levels of 10, 30 or 100 mg/kg/day.

Relevant findings

There were no clinical signs of toxicity, no mortalities, no treatment-related findings at necropsy.

An initial reduction in weight gain (and food consumption) on day 6 was resolved by day 11. Weight gain was not affected otherwise.

The uterine/implantation data were unaffected by the administration of pydiflumetofen. There was no effect of treatment on mean foetal, litter or placenta weights.

Some major abnormalities were noted

Major foetal abnormalities (table below) were noted in:

- 1 foetus from the control group
- 1 foetus from the 10 mg/kg/day group
- 2 foetuses from two litters in the 30 mg/kg/day group
- 2 foetuses from one litter in the 100 mg/kg/day group.

Table. *Summary of major foetal abnormalities*

Dose (mg/kg/day)	Dam	Fetus	Findings
0	16	L5	Diaphragmatic hernia
10	48	L8	Multiply malformed fetus
30	66	L4	Anophthalmia; oribital cavity reduced in size; malformed cervical neural arch; absent cervical neural arches; bent scapula
30	69	L12	Scapulae severly bent; humerii malformed; femurs bowed
100	82	L1	Exencephaly; open eye; cleft palate; malformed inter-parietals, parietals, frontals and nasals; cleft palatine
		R3	Exencephaly; open eye; malformed palate; malformed parietals and frontals; absent inter-parietals; cleft palatine

Controls: Diaphragmatic hernia was seen in one control foetus.

10 mg/kg bw/day: The one affected foetus had multiple severe malformations both external and internal including skeletal and sort tissues. This is considered to be a spontaneous malformation.

30 mg/kg be/day: 2 foetuses/2 litters with multiple malformations. Specific HCD were not presented for anophthalmia; oribital cavity reduced in size; malformed cervical neural arch or absent cervical neural arches. However, there were no additional occurrences in the 100 mg/kg bw/day dose level or in the preliminary study up to 1000 mg/kg bw/d.

Following a request from EFSA (February 2018), additional HCD have been submitted by the applicant (Crl: CD (SD) rat data (19 studies) from pre-natal developmental toxicity studies after consolidation of other contract research organisations (CRO's) under Charles River Laboratories

since 2016). Bent scapula was identified as a major abnormality which can occur as a background finding (3/337 litters).

100 mg/kg bw/day: Multiple malformations of the skull and oral cavity including cleft palate and exencephaly were seen in two foetuses of a single litter. Some of the malformations observed were compared to the HC data (which were considered relevant by the RMS (*Davies 2015*) for litter incidence in cleft palate/ malformed palate/exencephaly/open eye in studies performed between 2008 and 2012 (table below); cleft palate, exencephaly and open eye were within the range of the background data (*Davies, 2015*).

Table. *Summary of major foetal external abnormalities*

Observations	Type	Dose levels (mg/kg bw/day)				HCD range: incidence (group mean %)		
		0 (control)	10	30	100	Sequani [a]	CR Horsham [b]	CR Montreal [c]
External examination								
No. of foetuses (F)		*311*	*271*	*317*	*296*			
No. litters (L)		*24*	*21*	*24*	*23*			
Head: eye- uni- or bilateral: open	Major	F: 0 (0%) L: 0 (0%)	F: 0 (0%) L: 0 (0%)	F: 0 (0%) L: 0 (0%)	F: 2 (0.7%) # L: 1 (4.3%)	F: 0-1 (0-0.4%) L: 0-1 (0-5.3%)	F: 0-1 (0-0.4%) L: 0-1 (0-5.6%)	F: 0-2 (0-0.32%) L: 0-2 (0-4.2%)
Brain: exencephaly	Major	F: 0 (0%) L: 0 (0%)	F: 0 (0%) L: 0 (0%)	F: 0 (0%) L: 0 (0%)	F: 2 (0.7%) # L: 1 (4.3%)	-	F: 0-1 (0-0.3%) L: 0-1 (0-5%)	F: 0-2 (0-0.36%) L: 0-2 (0-5%)
Oral cavity: palate: cleft or malformed	Major	F: 0 (0%) L: 0 (0%)	F: 1 (0.3%) L: 1 (4.8%)	F: 0 (0%) L: 0 (0%)	F: 2 (0.67%) # L: 1 (4.3%)	F: 0-1 (0-0.4%) L: 0-1 (0-5.3%)	F: 0-1 (0-0.3%) L: 0-1 (0-4.2%)	F: 0-4 (0-0.34%) L: 0-4 (0-4.76%)
Runted foetus	Minor	F: 0 (0%) L: 0 (0%)	F: 0 (0%) L: 0 (0%)	F: 1 (0.3%) L: 1 (4.2%)	F: 0 (0%) L: 0 (0%)	-	-	-

Two foetuses from the same litter present malformations of the oral cavity (cleft palate/ malformated palate, cleft palatine) associated with malformations of the head (inter-parietals, parietals, frontals and nasals), exencephaly and open eye

However, further HCD on exencephaly in the Crl:CD (SD) rat was submitted during Peer Review on request from EFSA. This HCD demonstrated that the spectrum of malformations apparent in the pydiflumetofen prenatal developmental toxicity study in the rat at both 30 or 100 mg/kg bw/day are considered to be spontaneous in origin and have been observed for this strain in control females at similar incidences within the historical control data, and therefore, are considered to incidental to pydiflumetofen administration (see the highlighted in the table above).

Table. *A Comparison of Foetal Gross External Malformations from the Rat Developmental Toxicity Study with pydiflumetofen (Davies, 2015) and HCD Data in Crl: CD [SD] Rats.*

		Reference	**Sequani Limited, Ledbury**	**Charles River Laboratories, Montreal**	**Charles River Laboratories, Lyon**	**Charles River Laboratories, Ashland**	**MARTA (MARTA, 1996)**	**Ema *et al.*, 2014**
		Year	2007 to 2015	2007 to 2017	2012-2017	2016-2017	1988-1991	2001-2010
		Strain	Crl: CD [SD]	Crl: CD [SD]	Crl: OFA [SD]	Crl: CD [SD]	Crl: CD [SD]	Crl: CD [SD]
Malformation	**Study incidence**	**Dose (mg/kg bw/day)**						
Anophthalmia	1/21 litter 1/271 foetuses	30	0/478 litters 0/6108 foetuses	2/1462 litters 2/19277 foetuses	1/563 litters 1/2158 foetuses	0/288 litters 0/3628 foetuses	16/6102 litters 16/88270 foetuses	10†/5702 litters 10/39192 foetuses
Exencephaly	1/24 litters 2/296 foetuses	100	0/478 litters 0/6108 foetuses	1/1462 litters 1/19277 foetuses	1/563 litters 1/7079 foetuses	0/288 litters 0/3628 foetuses	16/6102 litters 26/88270 foetuses	7†/5747 litters 7/79960 foetuses
Open eye	1/24 litters 2/296 foetuses	100	1/478 litters 1/6108 foetuses	1/1462 litters 1/19277 foetuses	0/563 litters 0/7079 foetuses	0/288 litters 0/3628 foetuses	6/6102 litters 9/88270 foetuses	4†/5747 litters 4/79960 foetuses
Cleft palate	1/24 litters	100	2/478 litters	2/1462 litters	0/563 litters	1/288 litters	9/6102 litters	9†/5747 litters

	1/296 foetuses		2/6108 foetuses	2/19277 foetuses	0/7079 foetuses	3/3628 foetuses	9/88270 foetuses	9/79960 foetuses
Malformed palate (High arched palate)	1/24 litters 1/296 foetuses	100	1/478 litters 1/6108 foetuses	0/1462 litters 0/19277 foetuses	0/563 litters 0/7079 foetuses	0/288 litters 0/3628 foetuses	2/6102 litters 4/88270 foetuses	0/5747 litters 0/79960 foetuses
Malformed or absent nares	1/24 litters 1/296 foetuses	100	0/478 litters 0/6108 foetuses	1/1462 litters 1/19277 foetuses	0/563 litters 0/7079 foetuses	0/288 litters 0/3628 foetuses	5/6102 litters 5/88270 foetuses	4†/5747 litters 4/79960 foetuses

Exencephaly was reported to occur in this strain of rat and its occurrence in two foetuses from the same litter in the group given 100 mg/kg/day, but was not considered to indicate an adverse effect of treatment. **Note:** the background incidence of foetuses with multiple malformations as described is, however, not available.

A number of variations/abnormalities were significantly increased at 100 mg/kg bw/day.

- Absent costal cartilage (a skeletal abnormality/variation) was observed in rats (also seen in rabbits) ($p<0.05$) at 100 mg/kg/day, just within the available HCD.
- In the groups receiving 30 or 100 mg/kg/day, there was a significant ($p<0.05$) increase, compared with the control group, in the number of litters with foetuses showing 'small area of liver protruding into the thorax', which is considered to be a minor abnormality, as there is no hole in the diaphragm and there is no consequence on post-natal survival and development. This finding is a background lesion that occurs sporadically.
- Left sided umbilical artery was increased at this dose. The increase was within the HCD range provided.

Table. *Summary of minor foetal abnormalities and variants from external/ visceral/ skeletal examination (F: foetal incidence (group mean %); L: litter incidence (group mean %))*

Observation	**Dose levels (mg/kg bw/day)**				
	0	**10**	**30**	**100**	**HCD range from Sequani d/e (incidence (group mean %))**
Umbilical artery (left side)	F: 1 (0.6%) L: 1 (4.2%)	F: 2 (1.5%) L: 2 (9.5%)	F: 1 (0.6%) L: 1 (4.2%)	F: 6 (3.8%) L: **5 (22%*)**	F: 0-8 (0-5.4%) [e] L: 1-7 (12-32%)
Liver ≥ 1 lobe: small area protruding into the thorax	F: 0 (0%) L: 0 (0%)	F: 1 (1.0%) L: 1 (4.8%)	F: 4 (2.6%) **L: 4 (16.7%)***	F: 3 (2.1%) **L: 3 (13%)***	F: 0-3 (0-2.7%) [e] L: 0-3 (0-15.8%)
Rib ≥1 costal cartilage absent	F: 0 (0%) L: 0 (0%)	F: 0 (0%) L: 0 (0%)	F: 2 (1.2%) L: 2 (8.3%)	F: 2 (1.5%) L: 2 **(8.7%)***	F: 1-5 (0.7-1.6%) L: 1-2 (4.5 -9.5%)ǂ

The number of foetuses showing these minor or variant abnormalities was very small and within the background data ranges and their incidences do not indicate an adverse effect on foetal development.

The foetal malformations and variations seen in the rat main study lack a dose-response relationship and statistical significance when compared with the controls, and were not apparent in the previous preliminary dose-range finding study (*Anon., 2011*) at significantly higher doses of pydiflumetofen (100, 500 and 1000 mg/kg bw/d). This demonstrates a distinct lack of dose concordance; no major foetal abnormalities were observed at up to 1000 mg/kg bw/d in the preliminary rat study. These findings were not considered treatment related by the RMS.

3.2.1.3 Conclusion

RAC can agree with the argument of the RMS.

3.2.2 Rabbit data

3.2.2.1 Preliminary study

Preliminary study: 0, 250, 500 or 1000 mg/kg/day on days 6-27 of gestation in NZW rabbits.

The highest dose was well tolerated with a slight initial reduction in body weight gain (GD 6-12) ($p<0.05$ to $p<0.01$). No statistically significant effect on body weights was observed over the duration of the study (Days 6 to 28) after correction for gravid uterine weight.

Foetal effects

All pregnancy related endpoints were unaffected.

A number of malformations were recorded, the majority occurring in the controls. Two foetuses in treated groups had abnormalities of the head; one foetus in Group 2 (250 mg/kg/day) with cheilognathopalatoschisis (cleft lip, jaw and palate) and one foetus in Group 4 (1000 mg/kg/day) with proboscis, cyclopia and oral cavity and jaw abnormalities.

Table. *Summary of major abnormalities*

Dose (mg/kg/day)	Dam	Fetus	Findings
0	41	R1	Thoracogastroschisis; left forelimb amelia; facial cleft on left side; microencephaly, pinna right low set; pinna left anotia. Left eye anophthalmia; left kidney pelvic; ovary bilateral ectopic; heart – persistent truncus arteriosus; intraventricular septum absent; descending aorta enlarged severely.
	44	R5	Heart severely enlarged; transposition of the great vessels; intraventricular septum absent; both lungs sevelery reduced in size
	46	R1	Left pinna malformed; abdomen – fissure of body wall
	48	L6	Interrupted aortic arch
250	53	R2	Eye bilateral open; nares absent; cheilognathopalatoschisis; pinna bilateral low set.
	55	R1	Both lungs severely reduced in size.
1000	75	R4	Proboscis; cyclopia; nares absent; oral cavity reduced opening; agnathia; malformed palate; microglossia. Interrupted aortic arch.

Although very rare, these abnormalities were considered by the RMS/DS to be isolated incidences and unrelated to treatment.

3.2.2.2 Main study

Administration of pydiflumetofen, once daily, by oral gavage, to pregnant New Zealand White rabbits from GD 6 to Day 27, inclusive, at dose levels of 10, 100 or 500 mg/kg/day was well tolerated with no clinical signs, no effect on body weight or pregnancy.

Foetal effects

Major foetal abnormalities were noted in 5/3 foetuses/litters in the control group, 4/3 foetuses /litters in the group given 10 mg/kg/day, 3/3 foetuses/litters in the group given 100 mg/kg/day and in 2/2 foetuses/litters in the group given 500 mg/kg/day.

Malformations related to skeletal and neural arches were observed in 2 foetuses at 100 mg/kg/day and were also observed in one foetus at 500 mg/kg/d (table "Summary of major foetal abnormalities" below). However, all these skeletal malformations are within the HCD provided by the conducting laboratory (13 studies performed between 2009 to 2013, table " Summary HCD for malformations related to skeletal and neural arches", below)

Table: *Summary of major foetal abnormalities*

Dose (mg/kg/day)	Dam	Fetus	Findings
0	8	R3	Flattened right maxillary region of the head; malformed forelimbs; arthrogryposis; malrotated hindlimbs; enlarged right orbital cavity
	17	R1	Severely enlarged aortic arch; transposition of the great vessels, absent intraventricular septum
	22	L3	Severely enlarged aortic arch
		R1	Pulmonary valvular artesia; absent intraventricular septum
		R3	Duplicated sternebrae; sternal and xiphoid cartilage duplicated on 1st and 4th sternebrae
10	34	L1	Interrupted aortic arch; incomplete intraventricular septum
		R1	Interrupted aortic arch; absent intraventricular septum; severely enlarged pulmonary arch, entire heart & superior vena cavas; severely fused 1st to 6th sternebrae
	38	R6	Severely enlarged aortic arch
	46	R5	Pulmonary valvular artesia
100	61	L1	Malrotated fore-limbs; severely bent scapula; bowed radii, ulna, tibia and fibula
	65	L4	Malformed and discontinuous lumbar cord; malrotated hidlimbs; filamentous tail; centrally placed kidneys; undescended testes; 10 thoracic vertebrae; 10 pairs of ribs; 10th centra absent; 10th neural arches malformed & fused; absent lumbar sacral & caudal vertebrae
	72	L3	Spina bifida; bifid 7th lumbar to 4th sacral neural arches; malformed 1st to 4th sacral cartilaginous spinous processes; severely fused 6th to 8th caudal centra
500	76	R2	Severely fused 4th to 5th thoracic centra; 4th & 5th right ribs arising drom the same neural arch
	80	L1	Diaphragmatic hernia; small lungs; right sided descending aorta.

Table: *Summary HCD for malformations related to skeletal and neural arches*

External and fresh examination: other major abnormalities

Observations	Dose levels (mg/kg bw/day)				HCD Sequani from the conducting laboratory[#] Mean % (range)	
	0	10	100	500	N = 13 studies (2009-2013)	
Spina bifida	F: 0 (0%)	F: 0 (0%)	**F: 1 (0.7%) L: 1 (5.3%)**	F: 0 (0%)	F: 0.03% (0-0.7%) L: 0.41 (0-5.6%)	
Severely fused 4th to 5th thoracic centra	F: 0 (0%)	F: 0 (0%)	F: 0 (0%)	F: 1 (0.6%) L: 1 (4.8%)	Thoracic centra: one or more: major fusion	F: 0.11% (0-0.7%) L: 0.97% (0-5.3%)
Bifid 7th lumbar to 4th sacral neural archs	F: 0 (0%)	F: 0 (0%)	**F: 1 (0.7%) L: 1 (5.3%)**	F: 0 (0%)	lumbar neural arch: one or more :bifid	F: 0.03% (0-0.7%) L: 0.48% (0-5.6%)
4th & 5th right ribs arising from the same neural arch	F: 0 (0%)	F: 0 (0%)	F: 0 (0%)	**F: 1 (0.6%) L: 1 (4.8%)**	Ribs: one or more: arising from same neural arch	F: 0.05% (0-0.7%) L: 0.48% (0-5.3%)
Absent lumbar sacral & caudal vertebrae	F: 0 (0%)	F: 0 (0%)	**F: 1 (0.7%) L: 1 (5.3%)**	F: 0 (0%)	Sacral centra: absent Caudal vertebrae: absent	F: 0.06% (0-0.7%) L: 0.48 (0-5.3%) F: 0.06% (0-0.6%) L: 0.48 (0-4.8%)

Diaphragmatic hernia: The increased incidence of diaphragmatic hernia observed in one foetus at 500 mg/kg/d (1/154 (0.6%)) is well within the range of the HCD (0-1.6% (liver diaphragmatic hernia); 0-1.3% (stomach diaphragmatic hernia), see table below. The DS is of opinion that this finding may be considered incidental and not treatment related.

Table: *Diaphragmatic hernia*

Observations	Dose levels (mg/kg bw/day)				HCD Sequani from the conducting laboratory[a] Mean % (range)
	0	10	100	500	N = 36 studies (2010-2017)
No. of foetuses (F)	*163*	*142*	*132*	*154*	*3277*
No. litters (L)	*22*	*18*	*19*	*21*	*385*
Liver: diaphragmatic hernia	F: 0 (0%)	F: 0 (0%)	F: 0 (0%)	**F: 1 (0.6%)** **L: 1 (4.8%)**	F: 0.18% **(0-1.6%)** L: 1.6% **(0-14.3%)**
Stomach: diaphragmatic hernia	F: 0 (0%)	F: 0 (0%)	F: 0 (0%)	**F: 1 (0.6%)** **L: 1 (4.8%)**	F: 0.06% **(0-1.3%)** L: 0.5% **(0-11%)**

Skeletal variations: Costal cartilage variant: A marginally increased incidence of one cartilage variant (one or more costal cartilage interrupted (rib)) was observed in the groups given 100 mg/kg/day or 500 mg/kg/day compared with control. HCD for the costal cartilage variant from 54 rabbit prenatal developmental studies performed by the conducting laboratory were submitted and have been assessed by the DS. Taking into account limitation to a time-frame of +/- five years with respect to the completion date of the rabbit developmental study with pydiflumetofen (42 studies performed between 2007 to 2017), the increase at the highest dose of 500 mg/kg/day is within the spontaneous background data range for foetal incidence (8% vs 9.4%) and slightly above the range for litter incidences (47.6% *vs* 44.5%). At 100 mg/kg bw/day, the increase in both litter and foetal incidences is above the HCD ranges (table with variant skeletal findings with statistical significance). The litter is the unit of interest in reproductive and developmental toxicity studies and the highest litter incidence was 9 (44.5%) in 2/8 studies from the HCD. The litter incidence in both the 100 and 500 mg/kg bw/day was greater than this (12 (63%) and 10 (47.6%), respectively).

The possibility of a relationship to treatment cannot be excluded. However, costal cartilage variant can be considered spontaneous in origin in NZW rabbits. In addition, this finding is not defined as a malformation, but rather, as variations in cartilage development which do not impact normal growth or function. Attention is drawn to the absence of other associated changes in any rib parameters and the absence of a dose response relationship. There is no retardation of foetal growth and development associated with pydiflumetofen at ≥ 100 mg/kg bw.

3.3 Adverse effects on or via lactation

In the two generation reproduction study as described, the administration of 1500 ppm in the top dose group for females (equivalent to 116 mg/kg/day, F0; 141 mg/kg/day, F1) increased the relative liver weight in both generations. There were no clinical signs of toxicity, no mortalities and no treatment-related findings at necropsy, amounting to no evidence to suggest biologically significant maternal toxicity. There was no indication of impaired nursing behaviour or decreased pup viability during lactation and no effect on pup growth to weaning. The results of the study do not indicate any direct, adverse effect on the offspring due to transfer of the active substance via the milk or to reductions in the quality of the milk.

3.4 Comparison with the criteria

3.4.1 Consideration of Category 1A classification

According to the CLP criteria, classification in Category 1A is largely based on evidence from human data, which were not present in the CLH report. Therefore, classification as Repr. 1A is not warranted.

3.4.2 Consideration of Category 1B or 2 classification

Categories 1B and 2 are reserved for presumed and suspected human reproductive toxicants, respectively, and must be based on the presence of **clear** (Category **1B**) or **some** (Category **2**) evidence of alterations in sexual function, fertility, or development. In addition, the evidence for both hazard categories must be present in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of the other concurrent toxic effects.

3.4.2.1 Reproduction

Argument against classification

While the specific findings for sexual maturation in both sexes appear related to treatment, there was no effect on key downstream parameters of sexual function such as oestrus cycling, mating performance or general fertility and no effect on ano-genital distance. In addition, there was no effect observed on endocrine or reproductive organs in any of the available repeated toxicity studies (in rat, mice or dogs).

Argument for classification

Minimal parental toxicity was demonstrated when Wistar rats were administered pydiflumetofen up to 4500 ppm (277 mg/kg bw) in males and 1500 ppm (116 mg/kg bw/day) in females in a 2 generation study.

In the 2 generation rat study a statistically significant delay in vaginal opening without reduced body weight at the time was observed in females given 1500 ppm. A statistically significant delay in time to preputial separation in males at 4500 ppm was also observed along with a slight but not statistically significant reduction in body weight. Minor changes in postnatal body weight are not believed to account for this effect. There was no evidence to suggest the delays were caused by direct effects on the genital tract or by effects on systemic endocrine function. However, an association with treatment cannot be excluded; the effects, i.e. delays in the onset of both male and female puberty (data available in one generation only), may be regarded as a potential fertility retardation but it must be noted that these effects were observed in the absence of adverse effects on downstream reproductive performance, mating behaviour, conception, etc. Nevertheless, regulation 1272/2008 defines effects to be considered for classification in point 3.7.1.3 where it states that such adverse effects on sexual function and fertility include (but are not limited to) "…adverse effects on onset of puberty…". Another source of uncertainty comes from the fact that the maximal dose level tested could have been higher (particularly in females) than that proposed from the findings of the toxicokinetic and metabolic studies. Higher doses could potentially have led to more reproductive effects and so the available studies cannot fully inform on other potential reproductive effects by pydiflumetofen. Considering all of the available data and the limitations in the available studies, classification in Category 1B is not proposed on the basis of adverse effects on the onset of puberty alone.

All studies were technically conducted to guidelines but the data do not fully inform on all reproductive endpoints simply because of dosing shortcomings. The early culling of the F2 generation offspring means that data for pubertal onset is confined to a single generation of

animals. An argument can be made that higher doses should have been tested in both the 2 generation study and in the rat main developmental toxicity study for greater confidence and robustness of the data. Consideration was given to the fact that there were no downstream consequences on fertililty parameters in the 2-gen study and that no classification in a higher category could be supported. However, regulation 1272/2008 defines effects to be considered for classification in point 3.7.1.3 and mentions that adverse effects on sexual function and fertility include (but are not limited to) "…adverse effects on onset of puberty…". As a consequence, delays in the onset of puberty in two sexes may be considered as a case for Category 2 for fertility effects .

From a purely technical point of view pydiflumetofen satisfies the criteria for an adverse effect that is considered in CLP for classification purposes. However, central to all the decisions of RAC is the consideration of the weight of evidence of all the data available . In the case of pydiflumetofen, RAC considers that classification in category 2 for fertility is appropriate.

In summary, delayed pubertal effects were seen in both sexes, at the upper boundary of the HCD in the case of males which cannot be explained on the basis of body weight change alone, therefore these effects are considered treatment related, clearly impact on the development or time to attainment of puberty (which may potentially impact on human fertility or reproductive function) and therefore are considered sufficient for Category 2 classification.

3.4.2.2 Developmental toxicity

Table: *Summary of relevant data*

Method, guideline, deviations[1] if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results** **- NOAEL/LOAEL (for parent, offspring and for developmental effects)** **- target tissue/organ** **- critical effects at the LOAEL**	**Reference**
Developmental toxicity OECD 414 GLP Acceptable Oral (gavage) Rat, Crl:CD (SD) 24 mated females/group	PYDIFLUMETOFEN (SYN545974) (purity 98.5%) 0, 10, 30 or 100 mg/kg/day on days 6-19 of gestation Vehicle: 1% CMC (w/v)	*Maternal toxicity* 100 mg/kg/day: Initial reduction in BWG between first days of dosing (Day 6 to day 10). Maternal NOAEL 30 mg/kg/day *Developmental toxicity* Malformations and variations reported were not considered related to treatment 100 mg/kg/day: No effects at highest dose tested	*Anon.*, (2015)
Preliminary developmental toxicity Non-guideline Non-GLP Supplementary Oral (gavage) Rat, Crl:CD (SD) 6 mated females/group	PYDIFLUMETOFEN (SYN545974) (purity 98.6%) 0, 100, 200, 500 or 1000 mg/kg/day on days 6-19 of gestation Vehicle: 1% CMC (w/v)	*Maternal toxicity* 500 and 1000 mg/kg/day: Initial reduction of BWG during the first day of dosing (Day 6-7). *Developmental toxicity (no skeletal examination)* 1000 mg/kg/day: No effects at highest (limit) dose tested	*Anon.* (2011)
Developmental toxicity OECD 414 GLP Acceptable	PYDIFLUMETOFEN (SYN545974) (purity 98.5%) 0, 10, 100 or 500 mg/kg/day on days 6-27 of gestation	*Maternal toxicity* 500 mg/kg/day: No effects at highest dose tested Maternal NOAEL 500 mg/kg/day *Developmental toxicity*	*Anon.*, (2015b)

<table>
<tr><th>Method, guideline, deviations[1] if any, species, strain, sex, no/group</th><th>Test substance, dose levels duration of exposure</th><th>Results
- NOAEL/LOAEL (for parent, offspring and for developmental effects)
- target tissue/organ
- critical effects at the LOAEL</th><th>Reference</th></tr>
<tr><td>Oral (gavage)
Rabbit, New Zealand White
24 mated females/group</td><td>Vehicle: 1% CMC (w/v)</td><td><u>≥ 100 mg/kg/day</u>:
-malformations reported at all dose levels including controls were not considered related to treatment.
-Increased incidence of one skeletal variant (rib costal cartilage interrupted) without clear dose response but increased incidence above the HCD.
<table>
<tr><td></td><td colspan="4">Dose level (mg/kg/day)</td></tr>
<tr><td></td><td>0</td><td>10</td><td>100</td><td>500</td></tr>
<tr><td>Observations</td><td colspan="4">Rib: one or more: costal cartilage interrupted (variant)</td></tr>
<tr><td>Fetuses</td><td>8/163 (4.4%)</td><td>8/142 (5%)</td><td>14/132 (14%)</td><td>12/154 (8%)</td></tr>
<tr><td>Litters</td><td>6/22 (27.3%)</td><td>6/18 (33.3%)</td><td>12/19 (63%)*</td><td>10/21 (47.6%)*</td></tr>
</table>
HCD: foetus: 0-13 (0-9.4%) / Litters: 0-9 (0-44.5%)
Developmental NOAEL 10 mg/kg/day</td><td></td></tr>
<tr><td>Preliminary developmental toxicity
Non-guideline
Non-GLP
Supplementary
Oral (gavage)
Rabbit, New Zealand White
10 mated females/group</td><td>PYDIFLUMETOFEN (SYN545974) (purity 99.3%/ 98.5%)
0, 250, 500 or 1000 mg/kg/day on days 6-27 of gestation
Vehicle: 1% CMC (w/v)</td><td>Maternal toxicity
<u>1000 mg/kg/day</u>:
↓ body weight 35% days 6-28
Maternal NOAEL 500 mg/kg/day

Developmental toxicity (no skeletal examination)
Malformations reported at all dose levels including controls were not considered related to treatment
<u>1000 mg/kg/day</u>:
Developmental NOAEL 1000 mg/kg/day</td><td>Anon., (2015c)</td></tr>
<tr><td>Two generation reproduction
OECD 416
GLP
acceptable
Oral (continuous in diet)
Rat, Crl:WI (Han)
24/sex/group
(see also sections 2.6.3 and 2.6.6.3)</td><td>PYDIFLUMETOFEN (SYN545974) (purity 98.5%)
Males: 0, 150, 750 & 4500 ppm
Females: 0, 150, 450 & 1500 ppm
Continuous in the diet</td><td>Only data for offspring developmental toxicity are presented
<u>Offspring toxicity - Males</u>
<u>4500 ppm (364 mg/kg/day)</u>
F1: delayed sexual maturation (45.9 days versus 43.0 days in controls) concurrent with slight ↓ body weight
<u>Offspring toxicity - Females</u>
<u>1500 ppm (116 mg/kg/day)</u>
F1: delayed sexual maturation (33.0 days versus 30.3 days in controls). No subsequent effect on oestrus cycling, mating performance or fertility and no effect on ano-genital distance.</td><td>Anon., (2015)</td></tr>
</table>

<u>Arguments for classification/non-classification</u>

In the preliminary rat study, there were no adverse findings on development up to 1000 mg/kg bw/day. A number of foetuses with multiple malformations were observed sporadically in treated groups of the main study where exposure was up to 100 mg/kg bw/day. The findings were compared to a variety of sources of historical control data during the EFSA Peer Review and were generally within the ranges assessed.

In the rabbit, a considerable number of malformed foetuses were recorded across the doses from control to 1000 mg/kg bw. No particular dose or treatment-related pattern was apparent with

the exception of a rib variant (one or more costal cartilage interrupted) the incidence of which was elevated in foetuses/ litters from 100 mg/kg bw/day. The increase at the highest dose of 500 mg/kg/d was slightly above the range for litter incidences of the appropriate HCD (47.6% vs 44.5%). At 100 mg/kg bw/d, the increase in both litter and foetal incidences was above the HCD ranges (see table with variant skeletal findings with statistical significance above). The highest litter incidence of the HCD was 9 (44.5%) in 2/8 studies from the HC data. The litter incidence in both the 100 and 500 mg/kg bw/day was greater than this (12 (63%) and 10 (47.6%), respectively). The possibility of a treatment relationship cannot be excluded. However, costal cartilage variant can be considered spontaneous in origin in NZW rabbits. In addition, this finding is not defined as a malformation, but rather as variations in cartilage development and do not impact on normal growth or function. Classification for development is not proposed on the basis of these findings.

Conclusion

RAC considers that **classification in category 2 for fertility is appropriate**.

ENVIRONMENTAL HAZARD EVALUATION

RAC evaluation of aquatic hazards (acute and chronic)

Summary of the Dossier Submitter's proposal

The DS proposed to classify the substance as Aquatic Acute 1 – H400 (M=1) based on a 48 hours mean measured EC_{50} value of 0.12 mg/L for the freshwater amphipod *Hyalella azteca*, and as Aquatic Chronic 1 – H410 (M=1) based on lack of rapid degradation and a 32-d mean measured NOEC value of 0.025 mg/L for fish *Pimephales promelas*.

Degradation

Pydiflumetofen was stable to hydrolysis at acidic, neutral and alkaline pH conditions at 50°C.

Aqueous photolysis of pydiflumetofen was studied in pH 7 buffer (direct photolysis) and in natural water (indirect photolysis). Pydiflumetofen was degraded, primarily by dechlorination and phenyl ring degradation to produce phenyl-hydroxylated metabolites, carboxylic acid metabolites and carbon dioxide. Estimated DT_{50} values were 93 and 35 days (summer sunlight 30-50°N) in pH 7 buffer and natural water, respectively. No photo-degradates reached levels ≥ 5% Applied Radioactivity (AR) via direct photolysis. Photolysis in natural water led to the formation of the degradation products SYN548261 at ≥ 5% AR at two consecutive sampling intervals (maximum 7.3% AR after 21 days) and NOA449410 at a maximum level of 5.8% AR by the end of the experimental period (30 days).

Pydiflumetofen was not considered readily biodegradable under the conditions of the available 28-d ready biodegradability test (OECD TG 301F).

The aerobic mineralisation and degradation of pydiflumetofen in surface water was determined in the laboratory under dark conditions and light/dark conditions. No significant degradation of the substance was observed throughout the study. Mineralisation was low (< 1%) in all systems tested. DT_{50}s were extrapolated beyond the study period in all incubation groups and ranged from 637 to >1000 days for dark incubation and from 402 to 662 days for light/dark incubation.

The rate and route of degradation of [14C]-pydiflumetofen has been investigated in two water-sediment systems under laboratory aerobic and anaerobic conditions in the dark. In the aerobic systems 70 - 74% of applied pydiflumetofen remained in the total systems after 100 days (end of study). Only one metabolite was observed at levels above 5% AR and this was identified as SYN545547. It increased throughout the duration of the study and accounted for up to 12.3% AR in sediment extracts and 12.8% AR in the total system after 100 days. In the anaerobic systems, 54 - 64% of applied pydiflumetofen remained in the total systems after 100 days. As in the aerobic systems, the only metabolite exceeding 5% of applied radioactivity was SYN545547. It increased throughout the duration of the study and accounted for up to 26.5% AR in sediment extracts, 10.8% in water and 32.4% AR in the total system after 100 days.
The rate of degradation of pydiflumetofen and its metabolite SYN545547 in aquatic systems were assessed from the data from the aerobic water-sediment study according to FOCUS guidance on degradation kinetics (FOCUS 2006, 2011). The persistence endpoints for pydiflumetofen were $DegT_{50}$ of 270 - 299 days ($DegT_{90}$ of 976 - 1100 days) for degradation in the whole system and DT_{50} of 0.74 - 8.03 days ($DegT_{90}$ of 33.1 - 86.9 days) for dissipation in the water column. The modelling endpoints for pydiflumetofen ranged from 244 to 252 days (geometric mean $DegT_{50}$ of 248 days) for degradation in the whole system. For the metabolite SYN545547, persistence endpoints were $DegT_{50}$ of 18.6 - 455 days ($DegT_{90}$ of 61.9 - 1510 days). The modelling whole system degradation endpoints ranged from 18.6 to 455 days (geometric mean $DegT_{50}$ of 92.0 days).

The enantiomeric composition of pydiflumetofen in water was determined at the end of the aerobic mineralisation study, at the end of the aerobic and anaerobic incubations in water/sediment studies and at the end of the irradiation period in the water photolysis study compared to the ratio in the pydiflumetofen application solutions. The pydiflumetofen enantiomer did not change significantly over the course of these degradation studies.

The DS concluded that pydiflumetofen is not considered readily biodegradable. In addition, the results from hydrolysis and water-sediment studies show that pydiflumetofen is not degraded in the aquatic environment to a level > 70 % within a 28 days period. As a consequence, pydiflumetofen is considered not rapidly degradable for the purpose of classification and labelling.

Bioaccumulation

The experimentally derived Log K_{OW} of pydiflumetofen is 3.8 at 25°C (OECD TG 107).

A fish bioconcentration study (OECD TG 305, OPPTS 850.1730, GLP) is available for pydiflumetofen (Anonymous, 2014). Bluegill sunfish (*Lepomis macrochirus)* were exposed to a single concentration (4.9 µg/L) of the mixture of radiolabelled [Phenyl-U-^{14}C]-pydiflumetofen and unlabelled test substance for 19 days in a flow-through system, followed by a 7-d depuration period. The bioconcentration factors $BCF_{SS,\ lipid\text{-}normalised}$ and $BCF_{k,\ lipid\text{-}normalised}$ for whole fish were 31.1 and 189, respectively.

Based on the available data the DS concluded that pydiflumetofen has a low potential for bioconcentration and consequently does not meet the CLP criteria for bioaccumulation.

Aquatic toxicity

Reliable aquatic toxicity data are available for all three trophic levels, and a summary of the relevant information is provided in the following table (the key endpoints used in hazard classification are highlighted in bold). The results of the studies are expressed in terms of mean measured concentrations.

Table: *Summary of relevant information on aquatic toxicity*

Method/Exposure	Test organism	Endpoint	Toxicity values in mg/L	Reference
Short-term toxicity to fish				
OECD TG 203, OPPTS 850.1075, flow-through	*Lepomis macrochirus* (Bluegill sunfish)	96-h LC_{50}	0.48	Anonymous, 2014
OECD TG 203, OPPTS 850.1075, flow-through	*Cyprinus carpio* (Common carp)	96-h LC_{50}	0.33	Anonymous, 2013a
OECD TG 203, EC L142/446 C.1, OPPTS 850.1075, flow-through	*Oncorhynchus mykiss* (Rainbow trout)	96-h LC_{50}	0.18	Anonymous, 2012
OECD TG 203, OPPTS 850.1075, flow-through	*Pimephales promelas* (Fathead minnow)	96-h LC_{50}	0.35	Anonymous, 2013
OECD TG 203, OPPTS 850.1075, flow-through	*Cyprinodon variegatus* (Sheepshead minnow)	96-h LC_{50}	0.66	Anonymous, 2013b
Short-term toxicity to aquatic invertebrates				
OECD TG 202, EC L142/456 C.2, OPPTS 850.1010 Static	*Daphnia magna* (Water flea)	48-h EC_{50}	0.42	Fournier, 2012a SYN545974_10016
No specific guideline but OECD TG 202 was consulted. Static	*Asellus aquaticus* (Water louse)	96-h EC_{50}	4.21	Pickering, 2015 SYN545974_10305
No specific guideline but OECD TG 202 was consulted. Static	*Chaoborus crystallinius* (Phantom midge)	48-h EC_{50}	2.49	Joyce, 2015 SYN545974_10341
No specific guideline but OECD TG 202 was consulted Static	*Chironomus riparius* (Non-biting midge / Harlequin fly)	48-h EC_{50}	0.69	Joyce, 2015a SYN545974_10316 Pickering, 2015a SYN545974_10315
No specific guideline but OECD TG 202 was consulted Static	*Cloeon dipterum* (Mayfly)	48-h NOEC	5.01	Pickering, 2015a SYN545974_10315
No specific guideline but OECD TG 202 was consulted Static	*Crangonx pseudogracilis* (Freshwater amphipod)	48-h EC_{50}	1.23	Pickering, 2015b SYN545974_10306
No specific guideline but OECD TG 202 was consulted Static	*Cyclops agilis speratus* (Eastern oyster)	48-h EC_{50}	4.17	Joyce, 2015b SYN545974_10347
No specific guideline but OECD TG 202 was consulted Static	*Lumbriculus variegatus* (Blackworm)	48-h EC_{50}	4.65	Pickering, 2015c SYN545974_10304
No specific guideline but OECD TG 202 was consulted Static	*Lymnaea stagnalis* (Great pond snail)	48-h NOEC	7.3	Pickering, 2015d SYN545974_10303

Method/Exposure	Test organism	Endpoint	Toxicity values in mg/L	Reference
Short-term toxicity to fish				
OECD TG 202, OPPTS 850.1010 Static	*Hyalella azteca* (Freshwater amphipod)	48-h LC_{50}	**0.12**	Brougher *et al*. 2015 SYN545974_10354
OPPTS 850.1035, OPPTS 850.1000 Static	*Americamysis bahia* (Mysid)	96-h LC_{50}	0.16	Fournier, 2012b SYN545974_10015
OPPTS 850.1025 Static	*Crassostrea virginica* (Eastern Oyster)	96-h EC_{50}	0.31	Fournier, 2014a SYN545974_10099
Toxicity to algae and aquatic plants				
OECD TG 201, OPPTS 850.5400, EC 761/2009 C.3 Static	*Pseudokirchneriella subcapitata* (Freshwater Green Alga)	72-h E_rC_{50}	>5.9	Kirkwood, 2013 SYN545974_10013
		72-h E_yC_{50}	3.6	
		72-h E_bC_{50}	4.3	
OECD TG 201, OPPTS 850.5400 Static	*Skeletonema costatum* (Marine diatom)	72-h E_rC_{50}	2.7	Soucy, 2014 SYN545974_10105
		72-h E_yC_{50}	2.7	
		72-h E_bC_{50}	2.7	
OECD TG 201, OCSPP 850.4550 Static	*Anabaena flos-aquae* (Freshwater Blue-Green Alga)	72-h E_rC_{50}	3.6	Soucy, 2013 SYN545974_10091
		72-h E_yC_{50}	3.5	
		72-h E_bC_{50}	3.6	
OECD TG 201, OCSPP 850.4550 Static	*Navicula pelliculosa* (Freshwater Diatom)	72-h E_rC_{50}	1.6	Soucy, 2015 SYN545974_10097
		72-h E_yC_{50}	1.5	
		72-h E_bC_{50}	1.5	
OECD TG 221, OPPTS 850.4400 Semi-static	*Lemna gibba* (Duckweed)	7-d E_rC_{50}	> 6.3	Soucy, 2015a SYN545974_10088
		7-d NOE_rC	> 6.3	
Long-term toxicity to fish				
OECD TG 210, OPPTS 850.1400, EC L.142/603, C.15 Flow-through	*Pimephales promelas* (Fathead Minnow)	32-d NOEC (survival, mean length and mean dry weight)	**0.025**	*Anonymous*, 2015a
		32-d EC_{10} 32-d EC_{20} (Body length)	0.15 0.32	
		32-d EC_{10} (Body weight)	0.13	
OECD TG 210, OPPTS 850.1400 Flow-through	*Cyprinodon variegatus* (Sheepshead Minnow)	32-d NOEC (survival, mean length and mean dry weight)	0.17	*Anonymous*, 2015b
		EC_{10} (embryo hatching success)	0.34	
Long-term toxicity to aquatic invertebrates				
OECD TG 211, OPPTS 850.1300, EC L.142/674, C.20 Static-renewal	*Daphnia magna* (Water flea)	21-d NOEC (survival, reproduction, growth)	0.042	Fournier, 2015 SYN545974_10017
		21-d EC_{10} 21-d EC_{20} (survival)	0.094 > 0.31	
		21-d EC_{10} 21-d EC_{20} (reproduction)	0.085 0.13	
		21-d EC_{10}	0.21	

Method/Exposure	Test organism	Endpoint	Toxicity values in mg/L	Reference
Short-term toxicity to fish				
		21-d EC_{20} (body length)	> 0.31	
		21-d EC_{10} 21-d EC_{20} (dry weight)	0.16 0.20	
OCSPP 850.1350	*Americamysis bahia* (Mysid shrimp)	28-d NOEC (survival, reproduction, growth)	0.076	Sayers, 2015c SYN545974_10167
Chronic toxicity to algae or aquatic plant				
OECD TG 201, OPPTS 850.5400, OECD TG 201, OPPTS 850.5400, EC 761/2009 C.3 Static	*Pseudokirchneriella subcapitata* (Green alga)	72-h NOE_bC 72-h E_bC_{10} 72-h E_bC_{20}	0.9 1.0 1.4	Kirkwood, 2013 SYN545974_10013
		72-h NOE_yC 72-h E_yC_{10} 72-h E_yC_{20}	0.9 1.1 1.6	
		72-h NOE_rC 72-h E_rC10 72-h E_rC20	0.9 2.3 5.7	
OECD TG 201, OCSPP 850.4550 Static	*Anabaena flos-aquae* (Blue-green alga)	72-h NOE_bC 72-h E_bC_{10} 72-h E_bC_{20}	2.7 2.8 3.0	Soucy, 2013 SYN545974_10091
		72-h NOE_yC 72-h E_yC_{10} 72-h E_yC_{20}	2.7 n.d. 2.8	
		72-h NOE_rC 72-h E_rC_{10} 72-h E_rC_{20}	2.7 2.8 3.0	
OECD TG 201, OCSPP 850.4550 Static	*Navicula pelliculosa* (Diatom)	72-h NOE_bC 72-h E_bC_{10} 72-h E_bC_{20}	0.89 0.71 0.98	Soucy, 2015b SYN545974_10097
		72-h NOE_yC 72-h E_yC_{10} 72-h E_yC_{20}	0.89 0.68 0.97	
		72-h NOE_rC 72-h E_rC_{10} 72-h E_rC_{20}	0.89 0.97 1.1	
OECD TG 201, OPPTS 850.5400 Static	*Skeletonema costatum* (Marine diatom)	72-h NOE_bC 72-h E_bC_{10} 72-h E_bC_{20}	2.4 2.5 2.5	Soucy, 2014 SYN545974_10105
		72-h NOE_yC 72-h E_yC_{10} 72-h E_yC_{20}	2.4 2.5 2.5	
		72-h NOE_rC 72-h E_rC_{10} 72-h E_rC_{20}	2.4 2.5 2.5	
OECD TG 221, OPPTS 850.4400	*Lemna gibba*	7 day EC_{50} 7 day EC_{20}	>6.3	Soucy, 2015a

Method/Exposure	Test organism	Endpoint	Toxicity values in mg/L	Reference
Short-term toxicity to fish				
Semi-static	(Duckweed)	7 day EC_{10} (frond number)		SYN545974_10088
		7 day EC_{50} 7 day EC_{20} 7 day EC_{10} (dry weight)	>6.3	

Data for sediment-dwelling invertebrates (*Leptocheirus plumulosus*, *Chironomus dilutes* and *Hyalella azteca*) were reported in CLH report but were not used for classification because the endpoint values were presented in relation to sediment concentrations of pydiflumetofen (mg/kg). The only data for sediment-dwelling invertebrates available also in mg/L were the result of the acute toxicity study with freshwater amphipods *Hyalella azteca* which was used for classification of pydiflumetofen.

Acute toxicity

For fish, five studies were available. *Oncorhynchus mykiss* was the most sensitive fish species tested in the acute studies, with a 96-h LC_{50} of 0.18 mg/L.

Twelve studies were available in case of aquatic invertebrates. *Hyalella azteca* was the most sensitive species tested in the acute studies, with a 48-h LC_{50} of 0.12 mg/L.

Five acute toxicity studies were available for algae and aquatic plants. *Navicula pelliculosa* was the most sensitive species with a 72-h E_rC_{50} of 1.6 mg/L.

From the available aquatic toxicity data, invertebrates are the most sensitive trophic level therefore the acute aquatic classification proposed by the DS was based on freshwater amphipod *Hyalella azteca* (48-h LC_{50} = 0.12 mg/L). The DS proposed Aquatic Acute 1, with an M-factor = 1 (0.1 < LC_{50} < 1 mg/L).

Chronic toxicity

For fish, two studies were available. *Pimephales promelas* was the most sensitive fish species tested in the chronic studies, with a 32-d NOEC of 0.025 mg/L.

Long-term toxicity to aquatic invertebrates was assessed based on two available studies. The DS concluded that *Daphnia magna* was the most sensitive species tested in the chronic studies, with a 21-d NOEC of 0.042 mg/L. RAC is of the opinion that *Americamysis bahia* was the most sensitive invertebrate species tested in the chronic studies, with a 28-d NOEC of 0.076 mg/L (explanation provided in RCOM document).

Five chronic toxicity studies were available for algae and aquatic plants. *Navicula pelliculosa* was the most sensitive species tested in the chronic studies, with a 72-h NOEC of 0.89 mg/L. In the same study, the 72-h E_rC_{10} for *Navicula pelliculosa* (based on the same endpoint growth) was 0.97 mg/L.

The results of long-term aquatic toxicity studies indicate that the fish are the most sensitive taxon with a 32-d NOEC of 0.025 mg/L for *Pimephales promelas*. Therefore, the DS proposed

Aquatic Chronic 1, with an M-factor = 1 (0.01 < NOEC ≤ 0.1 mg/L) and the substance is not rapidly degradable.

Comments received during public consultation

Six MSCAs submitted comments on the environmental part of the DS's proposal during the public consultation (PC). Four MSCAs agreed with proposed classification for pydiflumetofen as Aquatic Acute 1, M-factor = 1 and Aquatic Chronic 1, M-factor = 1. One of them considered that the classification should be based on the surrogate approach for the most acutely sensitive endpoints. Two MSCAs indicated minor editorial mistakes in the CLH report.

One MSCA was of the opinion that the results from the study with marine diatom *Skeletonema costatum* (Soucy, 2014) and freshwater blue-green algae *Anabaena flos-aquae* (Soucy, 2013) should be considered as supplementary information because the coefficient of variation of mean daily growth rate in controls is above the validity criteria. In response, the DS stated that all other validity criteria were fulfilled and that the test was considered valid and relevant, and was not challenged during the peer review of the active substance. Moreover those algae studies are not the key studies for acute and chronic environmental hazard classification as algae are not the most sensitive aquatic organisms. Therefore, they do not impact on overall conclusion for classification.

The second commenting MSCA provided general comments regarding missing information in the combined CLH/DAR report (e.g. reliability and validity of the studies, etc.).

The third commenting MSCA pointed out that the bioaccumulation has been addressed for the active substance but no mention is made of the water metabolites SYN548261 (photolysis, max 7.3 % AR), NOAA449410 (photolysis, max 5.8 % AR), SYN545547 (aerobic, max 12.3% AR in total system). The DS responded that pydiflumetofen has a low potential for bioaccumulation and is not rapidly degradable. The latter together with chronic toxicity data drives the proposal for environmental hazard classification. Therefore the comment has no impact on harmonised classification proposal for environmental hazard. RAC agrees with the explanation provided by the DS.

In the view of the fourth commenting MSCA, the EC_{10}/EC_{20} (mean dry weight and mean length) should be used, if available, in preference to the NOEC (survival, mean length and mean dry weight) for *Pimephales promelas*. The same MSCA also asked for the EC_{10}/EC_{20} values for survival, if available. In response, the DS confirmed that the EC_{10} was calculated only for "total length" and "dry weight". The NOEC was based on the "live, normal larvae at hatch" and was considered valid and relevant during the peer review process of the active substance.

The same MSCA also noted that the fish *P. promelas* was not the most acutely sensitive fish species and suggested that the surrogate approach should be considered using the 96-h LC_{50} for *O. mykiss* of 0.18 mg/L. This would result in Aquatic Chronic 1 (M-factor = 1). The DS considered that *P. promelas* and *O. mykiss* have acute sensitivity in the same order of magnitude and therefore chronic data on *P. promelas* were considered relevant for classification. RAC agrees with DS' explanation.

The MSCA also pointed out that a chronic endpoint is available for *Hyalella azteca* (42-d NOEC 7.6 mg/kg) but the study details are not presented to consider its reliability and the endpoint is based on spiked sediment. On this basis, the MSCA suggested that the surrogate approach should be considered using the 48-h EC_{50} of 0.12 mg/L for *H. azteca* and this would result in Aquatic Chronic 1, with M-factor = 1. The DS considered that *D. magna* and *H. azteca* have acute sensitivity in the same order of magnitude and therefore chronic data on *D. magna* were considered relevant for classification. Classification based on chronic study on *D. magna* would result in classification as Aquatic Chronic 1, with M-factor of 1. RAC is of the opinion that the

chronic data for *Americamysis bahia* (NOEC of 0.076 mg/L) should be used for classification instead of chronic data for *Daphnia magna* as selected by the DS (explanation provided below and in RCOM document). *A. bahia* has an acute sensitivity in the same order of magnitude as *H. azteca*, therefore it is appropriate to consider chronic data for *A. bahia* relevant for classification. This does not change the proposed classification.

The fifth commenting MSCA disagreed with selected NOECs for aquatic invertebrates and aquatic plants by the DS. The MSCA pointed out that in line with the current CLP Guidance the preference to the EC_{10} value over the NOEC value is given. This applies in cases where EC_{10}s are available for the same endpoint. In the view of the MSCA the following endpoint should be selected as the most critical one:

- The EC_{10} of 0.085 mg/L for *D. magna* (reproduction) instead of NOEC (survival, reproduction and growth) (0.042 mg/L). The EC_{10} (0.085 mg/L) is however higher than the NOEC for *A. bahia* of 0.076 mg/L (survival, reproduction and growth). For this study, EC_{10} values could not be derived and therefore the NOEC for *A. bahia* is the most critical chronic endpoint for aquatic invertebrates.
- The E_rC_{10} of 0.97 mg/L for *N. pelliculosa* instead of NOE_rC of 0.89 mg/L.

The DS responded that this does not change the initially proposed classification. RAC agrees with the commenting MSCA regarding selection of the most critical chronic endpoint for aquatic invertebrates and algae/aquatic plants.

Assessment and comparison with the classification criteria

Degradation

RAC agrees with the DS proposal that pydiflumetofen does not meet the criteria for rapid degradation following CLP criteria. Based on available hydrolysis, results obtained in a biodegradation study, aerobic mineralisation and degradation in surface water study (no significant degradation, low mineralisation (< 1%)), aerobic water/sediment system study (70-74% remained in the total systems after 100 days) RAC agrees with the DS' conclusion that available degradation information does not indicate that pydiflumetofen is ultimately degraded (> 70%) within 28 days (equivalent to a degradation half-life of < 16 days). Consequently, pydiflumetofen is considered to be not rapidly degradable for the purpose of classification under the CLP Regulation.

Bioaccumulation

RAC agrees with the DS that pydiflumetofen has no potential to bioaccumulate in aquatic organisms. The basis for this is that log K_{OW} value of 3.8 is below the CLP threshold of 4 and both measured fish BCF_{ss} and BCF_k values are below the CLP criterion of ≥ 500.

Aquatic toxicity

Aquatic acute toxicity data are available for all three trophic levels. The lowest reliable short-term aquatic toxicity value is a 48-h LC_{50} of 0.12 mg/L for the invertebrate *Hyalella azteca*. As this is below the threshold value of 1 mg/L, pydiflumetofen meets the criteria for classification as Aquatic Acute 1 (H400). Since this toxicity value is in the range of $0.1 < LC_{50} \leq 1$ mg/L, the acute M-factor is 1.

Chronic toxicity

RAC is of the opinion that in case of pydiflumetofen, adequate chronic toxicity data are available for all three trophic levels, although the data for the most acutely sensitive fish and invertebrate

species is not represented. Based on the most sensitive standard test organism data (32-d NOEC of 0.025 mg/L for fish *Pimephales promelas*), and lack of rapid degradability, the substance should be classified as Aquatic Chronic 1 with a chronic M-factor of 1 (as 0.01 < NOEC ≤ 0.1 mg/L). The same conclusion on chronic classification would be reached based on the surrogate approach using *Oncorhynchus mykiss* (96-h LC_{50} of 0.18 mg/L) or *Hyalella azteca* (48-h LC_{50} of 0.12 mg/L) and lack of rapid degradation.

In summary, based on the available data, RAC considers to classify pydiflumetofen as Aquatic Chronic 1 with M-factor of 1.

In conclusion, RAC supports the DS's proposal that pydiflumetofen should be classified according to CLP as:

Aquatic Acute 1 (H400), M-factor = 1

Aquatic Chronic 1 (H410), M-factor = 1

ANNEXES:

Annex 1 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 2 Comments received on the CLH report, response to comments provided by the Dossier Submitter and RAC (excluding confidential information).