Committee for Risk Assessment

RAC

Opinion

proposing harmonised classification and labelling
at EU level of

flumioxazin (ISO); *N*-(7-fluoro-3,4-dihydro-3-oxo-4-prop-2-ynyl-2*H*-1,4-benzoxazin-6-yl)cyclohex-1-ene-1,2-dicarboximide

EC Number: -
CAS Number: 103361-09-7

CLH-O-0000001412-86-276/F

Adopted

15 March 2019

15 March 2019

CLH-O-0000001412-86-276/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: **flumioxazin (ISO); *N*-(7-fluoro-3,4-dihydro-3-oxo-4-prop-2-ynyl-2*H*-1,4-benzoxazin-6-yl)cyclohex-1-ene-1,2-dicarboximide**

EC Number: **-**

CAS Number: **103361-09-7**

The proposal was submitted by the **Czech Republic** and received by RAC on **1 February 2018.**

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

PROCESS FOR ADOPTION OF THE OPINION

The Czech Republic 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: **Miguel A. Sogorb**

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	613-166-00-X	flumioxazin (ISO); *N*-(7-fluoro-3,4-dihydro-3-oxo-4-prop-2-ynyl-2*H*-1,4-benzoxazin-6-yl)cyclohex-1-ene-1,2-dicarboximide	-	103361-09-7	Repr. 1B Aquatic Acute 1 Aquatic Chronic 1	H360D H400 H410	GHS08 GHS09 Dgr	H360D H410		M=1000 M=1000	
Dossier submitters proposal	613-166-00-X	flumioxazin (ISO); *N*-(7-fluoro-3,4-dihydro-3-oxo-4-prop-2-ynyl-2*H*-1,4-benzoxazin-6-yl)cyclohex-1-ene-1,2-dicarboximide	-	103361-09-7	**Retain** Aquatic Acute 1 Aquatic Chronic 1 **Modify** Repr. 2	**Retain** H400 H410 **Modify** H361d	**Retain** GHS08 GHS09 **Modify** Wng	**Retain** H410 **Modify** H361d		**Retain** M=1000 M=1000	
RAC opinion	613-166-00-X	flumioxazin (ISO); *N*-(7-fluoro-3,4-dihydro-3-oxo-4-prop-2-ynyl-2*H*-1,4-benzoxazin-6-yl)cyclohex-1-ene-1,2-dicarboximide	-	103361-09-7	**Retain** Aquatic Acute 1 Aquatic Chronic 1 **Modify** Repr. 2	**Retain** H400 H410 **Modify** H361d	**Retain** GHS08 **Modify** Wng	**Retain** H410 **Modify** H361d		**Retain** M=1000 M=1000	
Resulting Annex VI entry if agreed by COM	613-166-00-X	flumioxazin (ISO); *N*-(7-fluoro-3,4-dihydro-3-oxo-4-prop-2-ynyl-2*H*-1,4-benzoxazin-6-yl)cyclohex-1-ene-1,2-dicarboximide	-	103361-09-7	Repr. 2 Aquatic Acute 1 Aquatic Chronic 1	H361d H400 H410	GHS08 GHS09 Wng	H361d H410		M=1000 M=1000	

GROUNDS FOR ADOPTION OF THE OPINION

RAC general comment

Flumioxazin (ISO) is an active substance used in plant protection products as an herbicide. It is used for the pre-emergence control of many annual broad-leaved weeds and some annual grasses. Flumioxazin has a harmonised entry in Annex VI of the CLP regulation for its toxicity to reproduction as Repr. 1B (H360D; may damage the unborn child). The dossier submitter (Czech Republic) submitted in 2013 a CLH proposal to remove this classification arguing that the mode of action for the developmental toxicity is not relevant for humans. However, RAC confirmed this classification in June 2014 for the following reasons:

- Although the proposed mode of action leading to developmental toxicity was considered plausible by RAC, it was not convincingly explored at doses where developmental effects were in fact observed;
- Other modes of action could not be excluded;
- Regardless of the quantitative differences between rats and humans, the relevance for humans could not be totally ruled out.

Therefore, the manufacturer (Sumitomo Chemical Co., Ltd) conducted new studies to clarify the mode of action at appropriate doses in developmental toxicity studies in rats and to clarify the relevance of the mode of action to humans. This new CLH proposal (submitted by the Czech Republic but prepared by the manufacturer) discussed and summarised the key mechanistic work on mode of action and species differences in key events as well as assessed the weight of evidence against the CLP classification criteria.

HUMAN HEALTH HAZARD EVALUATION

RAC evaluation of reproductive toxicity

Summary of the Dossier Submitter's proposal

The dossier submitter (DS) proposed no classification of flumioxazin for fertility and sexual function since no treatment-related effects were observed in a two-generation study in rats.

For developmental toxicity, the DS proposed to change the classification of flumioxazin from Repr. Category 1B (H360D) to Repr. Category 2 (H361d) based on the following arguments:

Mode of action analysis

Mechanistic research previously established that haematotoxicity observed in the developmental studies and to a lesser extent in the repeat dose toxicity studies resulted from the inhibition of the enzyme protoporphyrinogen oxidase (PPO). PPO is responsible for the 7th step in haem production i.e. the step leading to the removal of hydrogen atoms from protoporphyrinogen IX to form protoporphyrin IX. The PPO inhibition therefore interferes with normal haem synthesis, which causes a reduction of red blood cells leading to embryo-foetal anaemia, embryolethality and the development of malformations, which included cardiac ventral septal defect (VSD), increased incidence of wavy ribs and reduced ossification of sacrococcygeal vertebral bodies. Rats are particularly sensitive to the effects of PPO inhibition induced by flumioxazin in

erythroblasts. This leads to anaemia that is a critical precursor of the developmental toxicity resulting from flumioxazin exposure.

According to the DS, a new mechanistic developmental toxicity study clearly demonstrated that the onset of foetal anaemia precedes the development of VSD at the lowest dose level at which such defects have been observed (30 mg/kg bw/d). It should therefore address the main concern of RAC that the proposed mode of action had only been demonstrated at a high dose and had not been explored at a lower dose where VSD defects were also observed. According to the DS, this new study also confirmed the finding of the previous developmental study that 30 mg/kg bw/d is the lowest observed effect level for a significant increase in the incidence of VSD.

Single mode of action

In the rabbit developmental study, whilst the administered dose was 100-fold greater than in the rat study and maternal toxicity was observed, no embryolethal or teratogenic effects were observed. The DS considered it as a convincing evidence for a single mode of action causing the developmental toxicity in the rat.

Relevance of the mode of action for humans

The DS concluded that in contrast to rats, humans are unlikely to develop anaemia resulting from inhibition of PPO. The DS based this conclusion on:

- Clinical findings that PPO deficient patients with variegate porphyria show no signs of anaemia, and moreover there are no reports of cardiac malformation in variegate porphyria patients or their babies.
- Experimental evidence that flumioxazin does not reduce haem synthesis in K562 cells and $CD36^+$ cells in vitro which are derived from human erythroleukemia and human cord blood, respectively.
- Experimental evidence *in vitro* showing that humans are less sensitive to PPO inhibition than rats. There is a species difference between rat and human in the erythropoiesis regulation during the developmental period. In the rat embryo, erythropoiesis begins with the production of a large synchronous wave of primitive erythroblasts over just a couple of days, which are released from the yolk sac into the circulation. This creates a very high demand for haem synthesis. The critical time for the rat foetus appears to be on GD12, when the primitive cells are almost all in the form of polychromatophilic erythroblasts. If these cells are damaged or killed, they cannot be replaced. Exposure to flumioxazin induces severe embryo anaemia and leads to hypoxia and VSD in rats. In humans, the primitive stages of erythropoiesis in the yolk sac occur over a much longer time period, which starts from the end of the second week of gestation and continues for several weeks until the foetal liver completely takes over production of red blood cells by 10-12 weeks of gestation (Ohls, 2011).
- New studies with dihydroartemisinin (DHA), which is an antimalarial drug, demonstrated a decrease in haem synthesis *in vitro* in both human-derived K562 cells and $CD36^+$ cells, and the potential to induce anaemia but, to date, there are no reports that DHA induces malformations in human foetuses. In clear contrast to DHA, flumioxazin does not inhibit haem synthesis *in vitro* in K562 cells or $CD36^+$ cells.

According to the DS, the results of the new studies are consistent with those of the former studies previously submitted by the manufacturer. The new studies demonstrate the reproducibility of the effects, confirm the mode of action for the developmental effects and show that the mode of action is unlikely to be of relevance for humans.

Pharmacokinetic differences between rats and humans

The pharmacokinetic modelling presented in the CLH report demonstrates that there is a marked kinetic difference between rats and humans. Human erythroblasts would not be susceptible to flumioxazin at exposure levels equivalent to a maternal dose exceeding 1000 mg/kg bw/d thus demonstrating the large species difference in sensitivity. In addition, as a result of the decrease in absorption rate with increasing oral dose, the systemic daily dose cannot exceed a value of approximately 100 mg/kg bw/d. Therefore, according to the DS, there is unlikely to be a plausible scenario whereby human exposure to flumioxazin could cause the developmental toxicity ascribed to the mode of action in the rat.

DS's conclusion

The DS concluded that the rat is not an appropriate model for assessing the developmental toxicity of flumioxazin in humans because, unlike humans, rats are highly sensitive to PPO inhibition, resulting in embryo/foetal anaemia and secondary developmental toxicity. A scenario whereby humans would be at risk of developmental toxicity given the species differences in susceptibility to the effects of flumioxazin on haem synthesis and the potential for anaemia is unlikely. Overall, the DS concluded that sufficient evidence is presented that raises doubt about the relevance of the effect for humans. Therefore, change of the current reproductive toxicity classification (Repr. 1B) is warranted and classification of flumioxazin as Repr. 2 is considered justified by the DS.

Comments received during public consultation

The manufacturer provided an attachment containing a new prenatal developmental toxicity study of flumioxazin in rats, a molecular simulation of PPO-flumioxazin interaction for insight into species difference, a study for determining the effects of flumioxazin on rat embryonic stem cell-derived erythroid cells and a study for determining the effects of flumioxazin on human induced pluripotent cells-derived erythroid cells.

One Member State Competent Authority (MSCA) provided comments noting that some of the original concerns raised by RAC in the opinion adopted in 2014 were addressed only to a limited extent and therefore it was difficult to assess whether the newly provided data sufficiently reduced the concern to allow a downgrade of the current classification to Category 2. The DS replied partly agreeing with the comments and reviewing most of the new information together with the information provided during the public consultation.

A second MSC disagreed with the DS proposal for downgrading the classification of flumioxazin. The main objections of this MSCA were: i) there was no clear demonstration that the rat erythroblast differentiation is really synchronised leading to a single population, instead of just happening faster than in humans; ii) it cannot be excluded that a repeated exposure during human early erythropoiesis would possibly lead to foetal anaemia due to a repeated impairment of the same targeted population; iii) the *in vitro* results should be considered with caution since they were obtained with erythroblast-like cancerous cells that might behave differently than normal erythroblasts to flumioxazin insults; iv) the *in vitro* assays should have been carried out with higher flumioxazin concentrations; and v) variegate porphyria is an autosomal dominant hepatic porphyria and in most of the cases, the enzyme inactivation is partial because only one allele is affected and the organism may compensate the slight impairment in haem production. Therefore, it is not expected to observe anaemia or developmental effects induced by foetal anaemia in variegate porphyria patients. The MSCA concluded that the mode of action is relevant for humans and the toxicokinetic differences between rats and humans are not so marked to assume that the hazard will not be expressed in humans. Therefore, taking into consideration

that teratogenicity was manifested in rats in the absence of maternal toxicity, and the human relevance, the MSCA considered the existing classification as Repr. Category 1B appropriate.

The DS answered by considering the newly available information submitted during public consultation: i) in humans, even if a particular population is affected, blood cell loss would not be as extensive as in rats; ii) the assumption that the same cells are exposed repeatedly may not be correct as in humans the erythroblasts are not all at the same level of differentiation at the same time; iii) 5 µM is the limit of solubility of flumioxazin and this concentration is much higher than the estimated human foetal exposure to flumioxazin following an *in vivo* maternal oral dose of 1000 mg/kg bw/d; iii) this maximum attainable concentration in biological media did not affect three different types of human erythroid cells; iv) the absence of anaemia in variegate porphyria patients, despite the reduction in PPO activity, are further indications that PPO is not a rate-limiting step in haem biosynthesis in humans. All these considerations allowed the DS to confirm that the case for reclassification into Repr. Category 2 is not based solely on the quantitative toxicokinetic differences between rat and human with respect to PPO inhibition. There is also a clear and consistent qualitative difference in the response to flumioxazin in the erythroid cells in rats and humans. Flumioxazin exposure did not inhibit the haem synthesis in any of three different types of human erythroid cells, in contrast to the rat where inhibition of haem synthesis, and consequent anaemia *in vivo* that caused the observed foetal effects, was observed.

Comments from four individuals or consultants commenting on behalf of the manufacturer supported the DS's proposal or no classification based on the lack of relevance of the mode of action for humans.

Assessment and comparison with the classification criteria

Fertility

The CLH-report contains a 2-generation reproduction toxicity study in rats. The main results of this study are summarised in the table below.

Table: *Summary table for the 2-generation reproduction toxicity study with flumioxazin****.***

Method	Results	Reference
2-generation study EPA OPP 83-4 GLP SD rats 30 rats/sex/dose Oral: feed 0, 50, 100, 200, 300 ppm Parental males: 0, 3.2, 6.3, 12.7, 18.9 mg/kg bw/d Parental females: 0, 3.8, 7.6, 15.1, 22.7 mg/kg bw/d F1 males: 0, 3.7, 7.5, 15.0, 22.4 mg/kg bw/d F1 females: 0, 4.3, 8.5, 17.2, 25.6 mg/kg bw/d Reliability: 1	<u>Parental toxicity</u> 300 ppm (males/females): Adverse clinical signs, reductions in body weight, body weight gain, food consumption and organ weights <u>Offspring toxicity</u> 200 ppm (males/females): Reduced pup body weights, increase in stillbirths with viability index and litter size reduced <u>Reproductive toxicity</u> 300 ppm (females): Reduced gestation index in both P1 and F1 generations and an increase in the number of F1 dams that did not deliver a litter	Hoberman, 1992 SBT-21-0035

In the 2-generation reproduction toxicity study, the following statistically significant adverse effects were reported (Table above): a) an increase in resorptions; b) a decrease in pups survival; c) a decrease in average pups weight; and, d) a reduction in gestation index. RAC considers these effects consistent with the increased embryolethality and growth retardation observed in the rat developmental studies with flumioxazin.

Development

Study examining effects of flumioxazin on the haem synthetic pathway and cell proliferation in rat erythroleukemia cells (Kawamura, 2013b; SBT-0125)

To investigate the effect of flumioxazin on the haem synthetic pathway, rat erythroleukemia cells (REL cell line) were induced to differentiate into erythroid cells by hexamethylenebisacetamide (HMBA). REL cells can differentiate into erythrocytes by treatment with various inducer chemicals such as HMBA. Concentrations of haem and protoporphyrin IX (PPIX) were determined after treatment of REL cells with HMBA and flumioxazin.

PPIX was accumulated in REL cells at 0.1 µM and above in a dose-dependent manner from day 2. The accumulation of PPIX reached a maximum at day 4. Haem synthesis was inhibited in REL cells at 0.1 µM and above in a dose-dependent manner from day 4. The inhibition of haem synthesis reached maximum at day 6. However, there was no effect on cell proliferation at the highest dose of 5.0 µM. The maximum accumulations of PPIX and maximum inhibition of haem synthesis are summarised in the table below.

Table: *Mean accumulation of PPIX and inhibition of haem synthesis in REL cells.*

[flumioxazin] (µM)	PPIX -day 4 (ng/10^6 cells) [% Control]	Haem-day 6 (ng/10^6 cells) [% Control]
0	0.63	127.06
0.01	0.60 [95]	116.09 [91]
0.1	1.11 [176]	91.49 [72]
0.3	1.94 [308]	85.11 [67]
1.0	5.95 [944]	59.72 [47]
5.0	14.04 [2222]	47.43 [37]

RAC notes that the PPO inhibition was also tested in other *in vitro* experiments with rat and rabbit cells. These studies allowed concluding that (see also the background document, under supplemental information):

- In the rat, flumioxazin is a stronger inhibitor (between 13.7 and 147 fold) than two hydroxylated flumioxazin metabolites; a third metabolite caused no inhibition of PPO up to 100 µM (Abe, 2011a, SBT-0118).
- Flumioxazin is about 13 fold stronger inhibitor of PPO in rat liver mitochondria than in rabbit liver mitochondria (Noda, 1995; SBT-0058).
- Adult liver and embryo mitochondria show similar sensitivity to PPO inhibition by flumioxazin in both rats and rabbits, with rabbit enzymes being less inhibited than rat enzymes (Green & Dabbs, 1993; SBT-31-0045).

Study examining effects of flumioxazin on the haem synthetic pathway and cell proliferation in human CD36+ cells (Kawamura, 2013a; study SBT-0126)

The human $CD36^+$ cells can differentiate into erythrocytes by culture with stem cell factor (SCF), erythropoietin (EPO), IL-3 and IL-6. To investigate the effect of flumioxazin on the haem synthetic pathway in human erythroid cells, human $CD36^+$ cells were cultured with SCF, EPO, IL-3 and IL-6 to differentiate into erythroid cells, and treated with flumioxazin.

PPIX accumulated in human $CD36^+$ cells at 1.0 µM and above in a dose dependent manner. However, there was no effect on cell proliferation or haem synthesis at the highest dose of 5.0 µM. The maximum accumulations of PPIX on day 8 and the corresponding data on haem synthesis are summarised in the table below.

Table: *Mean accumulation of PPIX and results of haem synthesis in CD36+ cells.*

flumioxazin (µM)	PPIX-day 8 (ng/10^6 cells) [% Control]	Haem-day 8 (ng/10^6 cells) [% Control]
0	1.18	1776.72
0.01	1.77 [150]	1706.27 [96]
0.1	1.59 [135]	2198.16 [124]
1.0	2.82 [239]	1882.45 [106]
5.0	13.97 [1186]	1534.81 [86]

RAC notes that other mechanistic *in vitro* studies were performed in order to establish the human relevance of the mode of action. These studies allowed concluding that (see also the background document, under supplemental information):

- Rat hepatocytes are more sensitive to flumioxazin treatment than human, rabbit and monkey hepatocytes (Abe, 2011b; SBT-0120).
- The relative sensitivity of the species to PPO inhibition by flumioxazin was rat > human > rabbit (Green & Dabbs, 1996; SBT-0060).
- PPIX accumulation in K562 cells was observed at concentrations of 1 µM and greater in a dose dependent manner without effect on cell proliferation or haem synthesis up to 5 µM (Kawamura, 2012a; SBT-0119). RAC also notes that the results summarised in Table 14 of the CLH report is consistent with the results of this study.
- There were no effect on PPIX content, haem synthesis or cell proliferation when K562 cells were treated with flumioxazin metabolites, while flumioxazin increased PPIX (Kawamura, 2012b; SBT-0123).
- A developed human physiologically based pharmacokinetic (PBPK) model demonstrated that the human foetal exposure to flumioxazin following a maternal oral dose of 1000 mg/kg would be 1.92 µM (Takaku, 2012b; SBM-0093)

Inhibition of protoporphyrinogen oxidase activity by flumioxazin and its major metabolites (3-OH flumioxazin, 4-OH flumioxazin and APF) in human liver mitochondria (Abe, 2014; SBT-0128) (NEW STUDY)

To investigate the inhibitory activity of flumioxazin and three major flumioxazin metabolites (3-OH flumioxazin, 4-OH flumioxazin and APF) against PPO, an enzyme inhibition assay was conducted *in vitro* using human liver mitochondrial fraction. The results of the inhibitory activity of the test substances are shown in the table below.

Table: *IC_{50} values of flumioxazin, 3-OH flumioxazin, 4-OH flumioxazin and APF against PPO in human liver mitochondria.*

Test substance	IC_{50} value (µM)			
	1st run	2nd run	3rd run	Mean
Flumioxazin	0.024	0.022	0.017	0.021
3-OH flumioxazin	0.126	0.097	0.089	0.104
4-OH flumioxazin	0.883	0.495	1.300	0.883
APF	ND	ND	ND	ND

ND: not determined.

The mean IC_{50} value of flumioxazin was 0.021 µM and it was almost the same as the previously reported IC_{50} value, 0.017 µM. Mean IC_{50} values of 3-OH flumioxazin and 4-OH flumioxazin were

0.104 µM and 0.893 µM i.e. 5 and 43 less potent than flumioxazin, respectively. An IC_{50} value for APF was not obtained in this study and was determined to be > 100 µM against human PPO.

RAC notes that the relative potencies of flumioxazin and its 3 major metabolites are broadly comparable with those obtained in rat liver mitochondria (SBT-0118), which suggests that metabolites of flumioxazin would have relatively weak or no significant PPO inhibitory activity in humans as well as rats.

Molecular simulation of PPO-flumioxazin interaction for insight into species difference (Arakawa et al., 2016; SBT-0135) (NEW STUDY)

A molecular dynamics *in silico* study to investigate the possible reasons for the species differences in the interactions between PPO and flumioxazin was submitted during public consultation. The molecular simulation showed that there are species differences in the dynamic behaviour of PPOs that affects binding energies. The difference in the dynamic behaviours between the three species was derived from the loop region of PPO with sequence variant. In the case of the human PPO-flumioxazin complex, the low binding affinity was due to weak van der Waals force associated with the dynamic behaviour of Arg-97. Whereas in the case of the rabbit PPO-flumioxazin complex, the low binding affinity was due to weak Coulomb force associated with dynamic behaviours of Phe-331 and Leu-334. These results support the case for PPO-based species differences in the inhibitory potency of flumioxazin, which is strongest in the rat and supports the lower sensitivity of humans to PPO inhibition.

Comparative effects of flumioxazin and dihydroartemisinin on the haem synthetic pathway and cell proliferation in rat erythroleukemia cells (Kawamura, 2015a; SBT-0132) (NEW STUDY)

Both flumioxazin and dihydroartemisinin (DHA) cause foetal anaemia, which leads to a similar pattern of developmental toxicity in the rat characterized by VSD and embryo/foetal death. Foetal anaemia from exposure to flumioxazin is caused by inhibition of haem synthesis resulting from inhibition of PPO. On the other hand, several hypotheses for the mechanism of foetal anaemia by DHA are reported.

To investigate the effect of flumioxazin and DHA on haem synthesis and PPIX accumulation in rat erythroid cells, a study was conducted with REL cells, which were induced to differentiate into erythroid cells by treatment with HMBA. It was concluded that exposure of differentiated REL cells to flumioxazin at a concentration of 5.0 µM resulted in (see also Figure 1):

- Accumulation of PPIX;
- Reduction of haem content per cell compared with controls; and,
- No effect on cell proliferation.

On the other hand, it was concluded that DHA caused:

- No PPIX accumulation;
- Dose-dependent reduction of haem content per cell at 0.5 µM and above with a maximum on day 6; and,
- Inhibition of cell proliferation at 0.5 µM and above in a dose dependent manner from day 2.

In conclusion, RAC notes that: 1) the results with flumioxazin are consistent with the results found in study SBT-0125; 2) flumioxazin causes a reduction of haem synthesis resulting from PPO inhibition but DHA inhibits haem synthesis by a different mechanism.

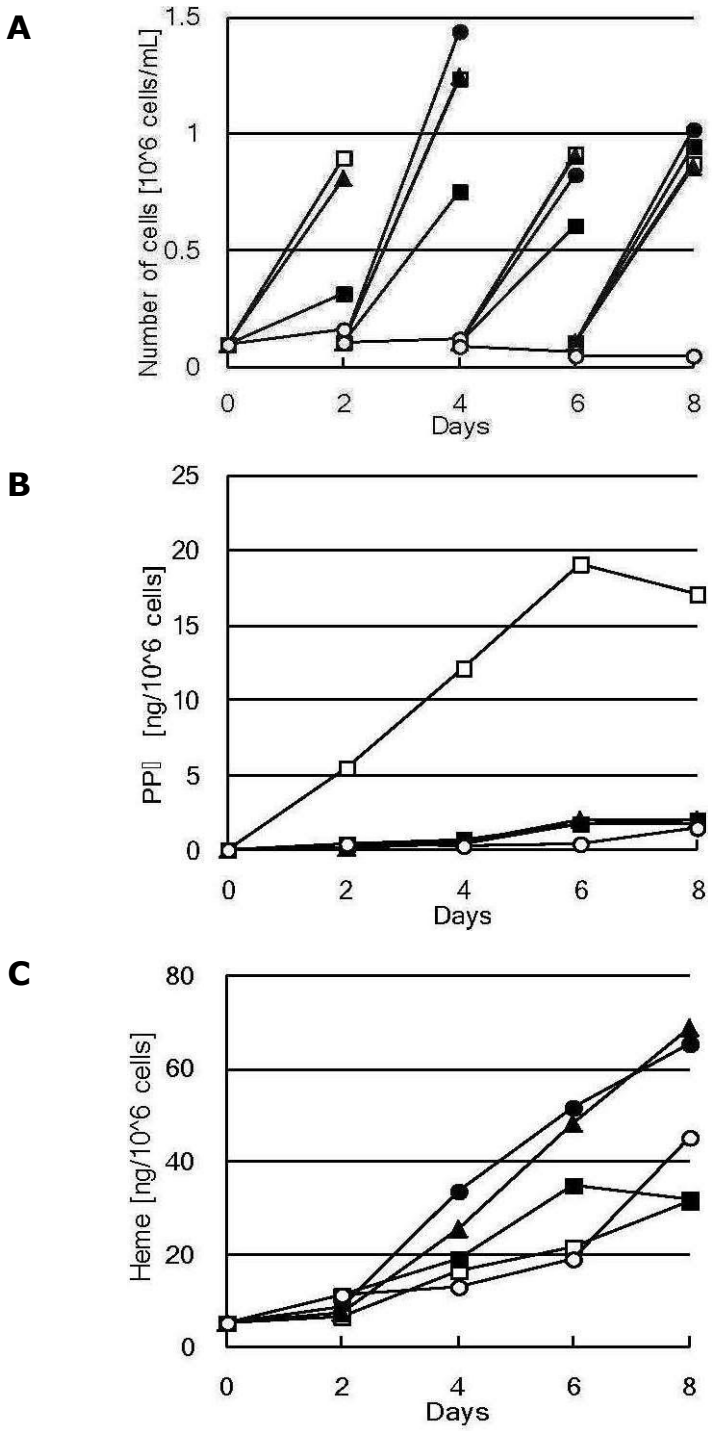


Figure 1: **Comparative effects of flumioxazin and DHA on cell proliferation (A), PPIX accumulation (B) and haem production (C) of differentiated REL cells.** 5.0 µM of flumioxazin (□), DHA concentrations: 0.125 µM (▲), 0.5 µM (■), 2.0 µM (○), and control (●).

Comparative effects of flumioxazin and dihydroartemisinin on the haem synthetic pathway and cell proliferation in human K562 cells (Kawamura, 2015b; SBT-0131) (NEW STUDY)

To investigate the effect of flumioxazin and DHA on haem synthesis and PPIX accumulation in human erythroid cells, a study was conducted with the human K562 cell line, which was induced to differentiate into erythroid cells by treatment with sodium butyrate (NaB).

It was concluded that exposure of differentiated K562 cells to flumioxazin at a concentration of 5.0 µM resulted in (see also Figure 2):

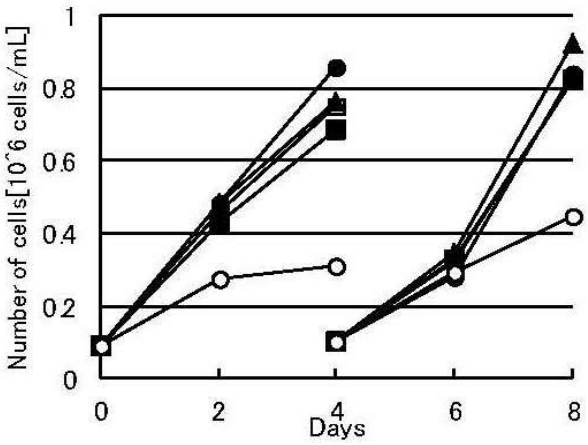
- accumulation of PPIX;
- no effect on haem content per cell compared with controls; and,
- no effect on cell proliferation.

On the other hand, it was concluded that DHA caused:

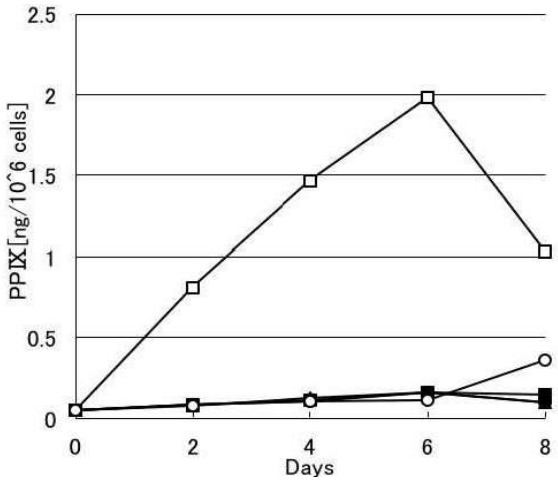
- no PPIX accumulation;
- reductions of haem content per cell at 20 µM with a maximum on day 6; and,
- inhibition of cell proliferation at 2.0 µM.

In conclusion, RAC notes that: 1) the results with flumioxazin are consistent with the results found in study SBT-0119 (see the background document, under supplemental information); 2) flumioxazin causes a reduction of haem synthesis resulting from PPO inhibition but DHA inhibits haem synthesis by a different mechanism.

A



B



C

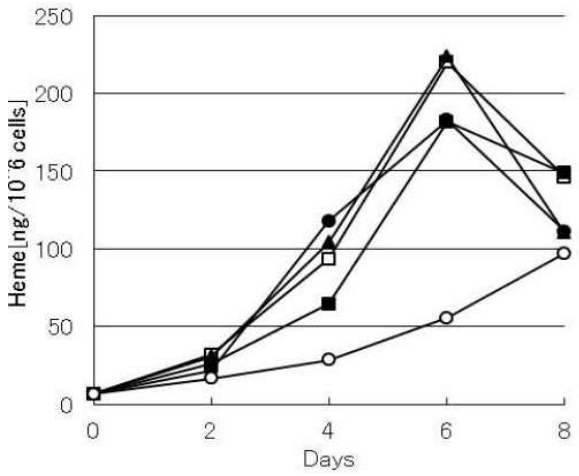


Figure 2: **Comparative effects of flumioxazin and DHA on cell proliferation (A), PPIX accumulation (B) and haem production (C) of differentiated K562 cells.** 5.0 µM of flumioxazin (□), DHA concentrations: 0.125 µM (▲), 0.5 µM (■), 2.0 µM (○), and control (●).

In summary:

- DHA caused inhibition of cell proliferation and reduction of haem content per cell in K562;
- DHA caused the inhibition of haem synthesis in REL cells (SBT-0132); and,
- flumioxazin inhibited haem synthesis in REL cells, but did not inhibit haem synthesis in K562 cells.

Overall, RAC notes a clear species difference in the inhibition of haem synthesis between rat and human haemoglobin synthesizing cells exposed to flumioxazin, in contrast to DHA for which no species difference was demonstrated**.**

Comparative effects of flumioxazin and dihydroartemisinin (DHA) on the haem synthetic pathway and cell proliferation in human CD36+ cells (Kawamura, 2015c; SBT-0130) (NEW STUDY)

In this study, the effect of flumioxazin and DHA on haem synthesis and PPIX accumulation was investigated in human $CD36^+$ cells, which are considered to be more biologically relevant than K562 cells. It was concluded that exposure of differentiated $CD36^+$ cells to flumioxazin at a concentration of 5.0 µM resulted in (see also Figure 3):

- accumulation of PPIX;
- no effect on haem content per cell compared with controls; and,
- no effect on cell proliferation.

On the other hand, it was concluded that DHA caused:

- no PPIX accumulation;
- dose-dependent reductions of haem content per cell at 0.125 µM and above with a maximum on day 8; and,
- inhibition of cell proliferation at 0.125 µM and above.

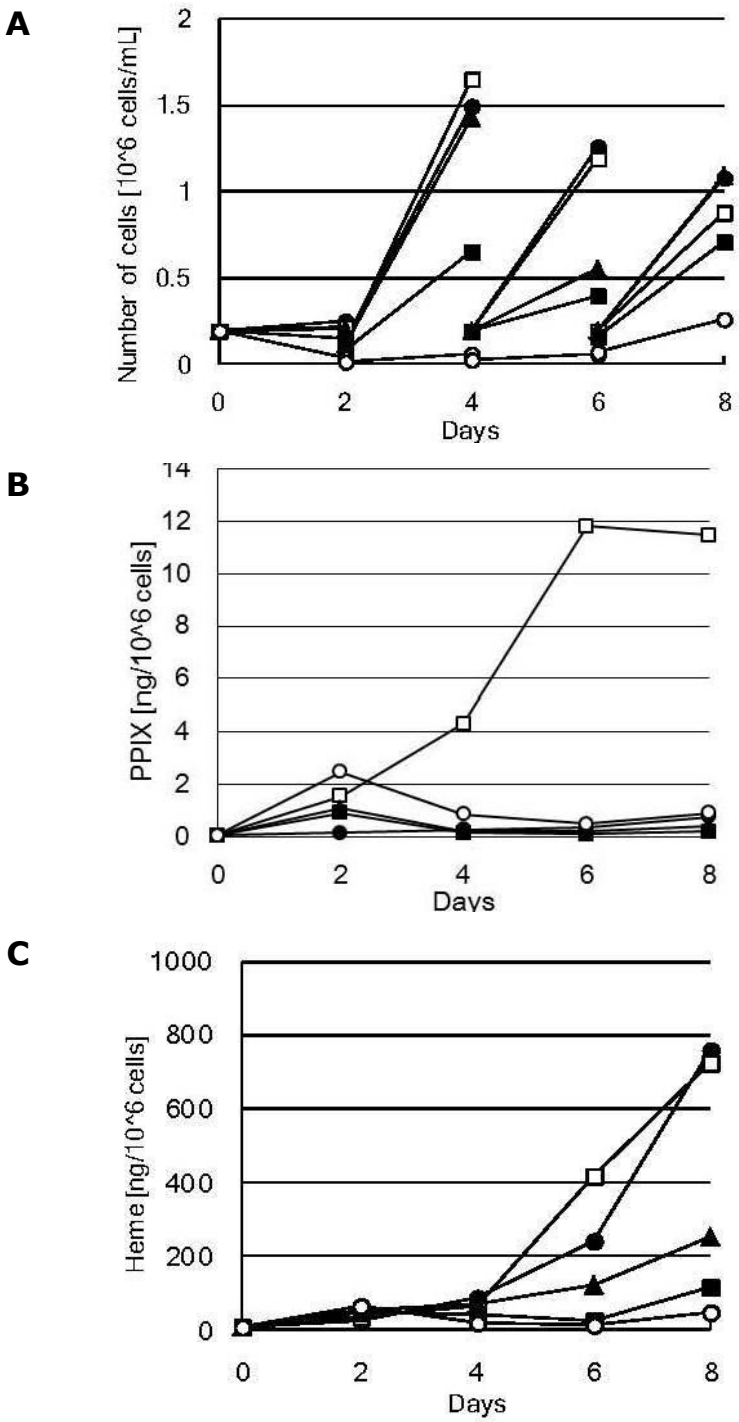


Figure 3: **Comparative effects of flumioxazin and DHA on cell proliferation (A), PPIX accumulation (B) and haem production (C) of differentiated CD36$^+$ cells.** 5.0 µM of flumioxazin (□), DHA concentrations: 0.125 µM (▲), 0.5 µM (■), 2.0 µM (○), and control (●).

In summary:

- DHA caused a reduction in haem content per cell in human CD36$^+$ cells with inhibition of cell proliferation and without PPIX accumulation.
- flumioxazin did not inhibit haem synthesis, did not affect cell proliferation and caused PPIX accumulation in human CD36$^+$ cells.

Overall, RAC notes that these results are consistent with those reported in SBT-0126 and with SBT-0131 and demonstrate that **DHA causes the inhibition of haem synthesis but flumioxazin causes no inhibition of haem synthesis in more biologically relevant human cells.**

Effects of flumioxazin on haem synthesis in human induced pluripotent cells-derived erythroid cells (Asano, 2018; SBT-0152) (NEW STUDY)

This study was submitted by the manufacturer during public consultation in order to evaluate the effects of flumioxazin and a positive control DHA on haem synthesis in a third type of human erythroid cell.

Most of the human induced pluripotent cells-derived erythroid cells were positive for glycophorin A (an erythroid marker protein) and embryonic ε-globin on differentiation day 10 and 14. Quantitative mRNA expression analysis showed that in human induced pluripotent cells-derived erythroid cells, over 60% of the total beta-like globin mRNA was embryonic ε-globin, confirming their similarity to human primitive erythroids. The remaining 28-38% was γ-globin, which is foetal globin but also expressed in primitive erythroid cells. In K562-derived erythroid cells, approximately 25-30% was ε-globin and the rest was γ-globin.

In the preliminary study, up to 0.5 µM DHA caused suppression of haem production, reduced cell proliferation and accumulation of PPIX on differentiation day 18. In contrast, there was no effect on haem synthesis or cell proliferation after treatment with flumioxazin at concentrations up to 5.0 µM. For the main study on erythroids derived from human induced pluripotent cells (hiPS) cells, the assay protocols were refined. In flumioxazin-treated erythroids, there was no effect on haem synthesis or cell proliferation, but PPIX was increased; in DHA-treated erythroids there was reduced haem synthesis, reduced cell proliferation, and no effect on PPIX (see Figure 4).

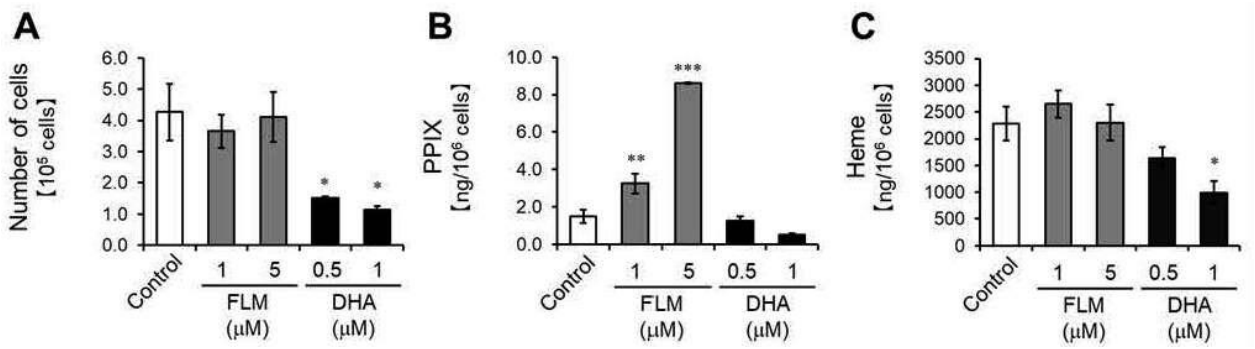


Figure 4: **Evaluation of the effects of flumioxazin and DHA on haem synthesis in erythroids derived from human induced pluripotent cells.** hiPS cell-derived erythroid cells were cultured in differentiation medium from differentiation day 10. Erythroid cells were exposed to flumioxazin, DHA or 0.1% DMSO (control). Erythroid cells were sampled at day 14 and the number of living cells were counted (A). Then, the cells were analysed for haem synthetic pathway products, PPIX (B) and haem content/concentration (C).
Values significantly different from control are: * $p<0.05$, ** $p<0.01$ and *** $p<0.001$. Abbreviations: DMSO, dimethyl sulfoxide; FLM, flumioxazin; DHA, dihydroartemisinin; PPIX, protoporphyrin IX.

Effect of flumioxazin on haem synthesis in rat embryonic stem cell-derived embryonic erythroid cells (Asano, 2018; BST-0163) (NEW STUDY)

This study used rat embryonic stem cells to generate embryonic erythroid cells and evaluated the effects of flumioxazin on haem synthesis in comparison with a positive control (DHA). Rat embryonic stem cells were differentiated into primitive erythroid cells by using hematopoietic-inducing medium and rat erythropoietin, which is involved in erythroid differentiation. After 8 days in culture, they were exposed to vehicle only, flumioxazin, or DHA. Haem synthesis, cell proliferation and PPIX accumulation were measured for 4 days in rat embryonic stem cell-derived embryonic erythroid cells.

Throughout the 8 days of differentiation, floating blood cells emerged. The pellets of produced cells showed dark red colour, which indicates a high level of haem synthesis. In addition, most floating blood cells expressed embryonic ε -globin, which is characteristic of embryonic erythroid cells. Moreover, quantitative mRNA expression analysis revealed that the globin expression level in rat embryonic stem cell-derived erythroid cells showed a high percentage (> 60%) of embryonic ε-globin thorough the 12 days differentiation. The high levels of ε-globin expression in erythroid derived from rat embryonic stem cells indicated that rat embryonic stem cell-derived erythroid cells could be considered as embryonic erythroid cells and are the closest to the rat primitive erythroid cells.

Haem synthesis was reduced and accumulation of PPIX was observed when treated with flumioxazin in a dose-dependent manner. DHA, the positive control, caused a reduction in cell proliferation and a reduction in haem synthesis in rat embryonic stem cell-derived embryonic erythroid cells (see Figure 5).

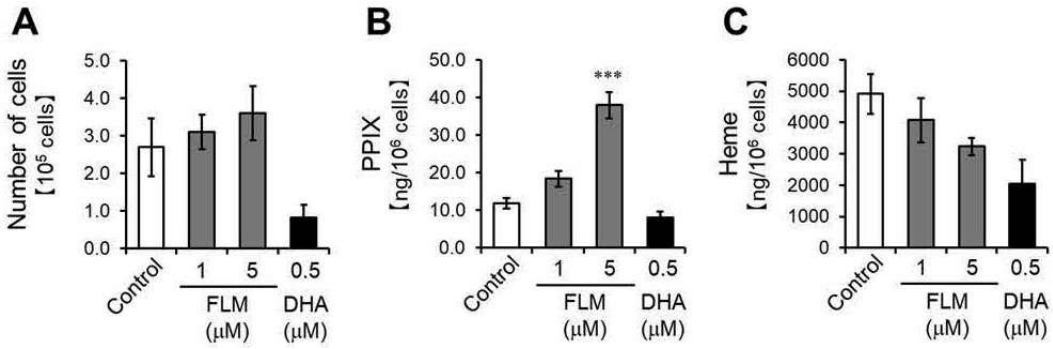


Figure 5: **Evaluation of effects of flumioxazin on haem synthesis in rat embryonic stem cell-derived embryonic erythroid cells.** Rat embryonic stem cells were cultured in differentiation medium for 8 days to produce embryonic stem cell-derived erythroid cells. These erythroid cells were exposed to flumioxazin, DHA or 0.1% DMSO (control). They were sampled at day 12 and the number of living cells were counted (A), and then analysed for PPIX (B) and haem (C). Values significantly different from control were: *** $p<0.001$. Abbreviations: DMSO, dimethyl sulfoxide, DHA, dihydroartemisinin, FLM, flumioxazin, PPIX, protoporphyrin IX.

RAC notes that these results, showing inhibition of haem synthesis in rat embryonic stem cell-derived embryonic erythroid cells treated with flumioxazin, are consistent with those previously reported using REL cells (SBT-0125, SBT-0132). In K562 cells (SBT-0131), $CD36^+$ cells (SBT-0126, SBT-0130) and human induced pluripotent stem cell-derived embryonic erythroid cells (SBT-0152), on the contrary, there were no effects on haem synthesis or cell proliferation, even when treated with 5 µM of flumioxazin, a concentration close to its water solubility limit.

RAC also notes a clear qualitative difference between human and rat erythroid cells in their response to flumioxazin, with no inhibition of haem synthesis in three different types of human erythroid cells seen in other studies but a reduction in haem synthesis in rat erythroid cells.

Additional study to evaluate the potential of flumioxazin to cause foetal anaemia at developmentally toxic doses in rats (SBT-0129, anonymous 2015) (NEW STUDY)

A previous study had shown that flumioxazin induces embryo/foetal lethality and teratogenicity (mainly VSD) when given orally at a dose of 30 mg/kg bw/d on gestation days 6-15 (SBT-00-0012) (see the background document, under additional key elements). A subsequent mechanistic study showed that a single oral dose of 400 mg/kg bw on gestation day 12 resulted in severe anaemia in the embryo and foetus, and that this is the most likely cause of the observed embryo/foetal lethality, enlarged heart and VSD (SBT-0065).

The objective of the present study was to assess whether anaemia also occurs in the embryo at the lower dose of 30 mg/kg bw/d, in order to better understand the cause of flumioxazin-induced embryo/foetal lethality and teratogenicity.

Flumioxazin (purity 99.6%) was administered to three groups of 20 mated (presumed pregnant) female Crl:CD(SD) rats as a single daily gavage dose from days 6 through 15 of gestation at dose levels of 15, 30 or 60 mg/kg bw/d. A concurrent control group of 20 pregnant females were similarly dosed with the vehicle only.

All maternal animals survived to the scheduled sacrifice on days 14 and 20 of gestation. Red fluid around the genital region observed in a few females of the treated groups were considered to be related to intrauterine embryo/foetal death at 30 and 60 mg/kg bw/d, but the single instance in the 15 mg/kg bw/d group was not considered to be treatment-related because there was no post-implantation loss in the dam.

The main results of the study are summarised below (see also table below):

- Maternal body weight and body weight gain were significantly decreased on day 20 of gestation at 60 mg/kg bw/d (this effect on maternal body weights was also considered to be due to intrauterine embryo/foetal death).
- No treatment-related effects on maternal body weight or body weight gain at 15 and 30 mg/kg bw/d.
- Necropsy of dams on days 14 and 20 of gestation revealed no treatment related macroscopic pathology findings.
- No dams with total litter resorptions by day 14.
- Four dams with total litter resorptions at 60 mg/kg bw/d by GD 20.
- The number of dams with live foetuses was 10, 9, 10 and 6 in the control, 15, 30 and 60 mg/kg bw/d groups, respectively.
- Embryolethality (post-implantation loss) was significantly increased at 60 mg/kg bw/d by day 14.
- No increase in embryolethality was observed in the 15 or 30 mg/kg bw/d groups.
- Paling of yolk sacs and embryos was increased significantly and dose-dependently at 30 and 60 mg/kg bw/d.
- Slight increases in pale yolk sacs and pale embryos at 15 mg/kg bw/d were not statistically significant.
- Iron deposits in erythroblasts were increased significantly and dose-dependently in the treatment groups but the increase at 15 mg/kg bw/d was very slight, which suggested that flumioxazin caused inhibition of haem synthesis at 30 mg/kg bw/d and above.

Table: *Selected maternal caesarean section and embryo data (day 14 of gestation).*

Parameter	Dose level (mg/kg bw/d)			
	0	**15**	**30**	**60**
Post-implantation loss (%):				
early	6.5	5.6	3.4	5.0
late	0.7	0.7	2.1	**12.8***
total	7.2	6.3	5.5	17.7
Number of live embryos per litter	12.8	13.4	13.8	11.6
Colour tone of yolk sacs (%):				
normal	97.7	88.1	**33.3****	**3.4****
pale	2.3	11.9	**59.4****	**67.2****
marked pale	0.0	0.0	**7.2****	**29.3****
Colour tone of embryos (%):				
normal	100.0	96.8	**49.5****	**5.3****
pale	0.0	3.2	**43.4****	**81.6****
marked pale	0.0	0.0	**7.1****	**13.2****
Iron deposits (granules) in erythroblasts (%)[1]:				

Parameter	Dose level (mg/kg bw/d)			
	0	15	30	60
no positive reaction	52.7	**40.8***	**54.2***	**46.2****
+	46.9	**53.7***	**30.4***	**27.2****
++	0.5	**5.3***	**11.6***	**22.3****
+++	0.0	**0.2***	**3.9***	**4.4****

Note: + a small number of positive granules scattered in the cytoplasm; ++ many fine positive granules in perinuclear cytoplasm; +++ massive deposition of positive granules. [1]200 erythroblasts examined per embryo. * Statistically different from control group p<0.05; ** statistically different from control group p<0.01.

The following histopathological changes were observed in the embryonic cardiovascular system and liver (see table below):

- Dose-dependent decreased contents of erythroblasts in the heart statistically significant at 60 mg/kg bw/d.
- Increased degenerative erythroblasts in the liver at 30 and 60 mg/kg bw/d.
- Thinning of the ventricular walls in the heart of embryos from the 30 and 60 mg/kg bw/d groups.
- Dilatation of the heart atrium in embryos from 60 mg/kg bw/d groups.
- Hepatocytic necrosis and dilatation of hepatic sinusoidal vessels at 60 mg/kg bw/d.

These results demonstrated that flumioxazin caused anaemia in the embryos on day 14 of gestation when administered orally to pregnant rats at 30 mg/kg bw/d and above from day 6 of gestation.

Table: *Histopathological findings in embryos (day 14 of gestation).*

Parameter	Dose level (mg/kg bw/d)			
	0	15	30	60
Heart-thin ventricular wall (%):				
normal	100	100	**65.0****	**25.0****
slight	0.0	0.0	**20.0****	**40.0****
mild	0.0	0.0	**15.0****	**30.0****
moderate	0.0	0.0	**0.0****	**5.0****
Heart-dilatation, atrium (%):				
normal	100	100	95.0	**80.0***
slight	0.0	0.0	0.0	**0.0***
mild	0.0	0.0	0.0	**20.0***
moderate	0.0	0.0	0.0	**0.0***
Heart-erythroblast content (%):				
abundance	75.0	81.0	50.0	**10.0****
depletion	25.0	19.0	40.0	**35.0****
marked depletion	0.0	0.0	10.0	**55.0****
Liver-degenerative erythroblasts (%):				
normal	100	95.2	**20.0****	**0.0****
slight	0.0	4.8	**75.0****	**75.0****
mild	0.0	0.0	**5.0****	**20.0****
moderate	0.0	0.0	**0.0****	**5.0****
Liver-dilatation of sinusoidal vessels (%):				
normal	100	100	100	**75.0****
slight	0.0	0.0	0.0	**15.0****
mild	0.0	0.0	0.0	**5.0****
moderate	0.0	0.0	0.0	**5.0****
Hepatocyte necrosis (peripheral regions) (%):				
normal	100	100	100	**80.0***
slight	0.0	0.0	0.0	**10.0***
mild	0.0	0.0	0.0	**5.0***
moderate	0.0	0.0	0.0	**5.0***

Note: * statistically different from control group p<0.05; ** statistically different from control group p<0.01. There was no severe grade for any of the histopathological findings.

In the 2014 RAC opinion, wavy ribs and delayed ossification of the ribs were attributed to the suppressed liver function resulting in reduction in protein synthesis. RAC notes that the liver damage is also reproduced in this new study.

On day 20 of gestation the following effects were noted (see table below):

- Increased embryolethality (post-implantation loss) at 60 mg/kg bw/d with no clear treatment related effect at 15 and 30 mg/kg bw/d.
- Significant decrease in foetal body weight in males and females of the 60 mg/kg bw/d with slightly but not statistically significant decrease at 30 mg/kg bw/d.
- Significant increase in the number of live foetuses with VSD at 30 and 60 mg/kg bw/d but not at 15 mg/kg bw/d.

Table: *Selected maternal caesarean section and foetal data (day 20 of gestation).*

Parameter	Dose level (mg/kg bw/d)			
	0	**15**	**30**	**60**
Post implantation loss (%):				
early	6.0	9.1	6.2	**61.2***
late	0.0	0.0	2.8	1.6
total	6.0	9.1	9.0	**62.8****
Number of live foetuses per litter:				
male	7.2	6.4	6.6	**2.2****
female	7.0	5.8	6.6	**2.6****
total	14.2	12.2	13.2	**4.8***
Foetal body weight (g):				
male	3.82	3.64	3.58	**3.34****
female	3.55	3.45	3.32	**3.11****
total	3.69	3.57	3.45	**3.17****
Visceral examination- number of dams with anomalous foetuses (%):	20.0	33.3	80.0	**83.3***
Number of foetuses with VSD (% of foetuses examined):	1.4	4.5	**19.7***	**37.5***

*statistically different from control group p<0.05; ** statistically different from control group p<0.01.

In conclusion, flumioxazin caused embryonic anaemia when administered orally to pregnant rats at 30 mg/kg bw/d and above from days 6-15 of gestation and the severe anaemia caused embryo/foetal deaths and VSD. The clear effect dose level for embryonic anaemia and foetal teratogenicity was 30 mg/kg bw/d and a small effect was seen at 15 mg/kg bw/d. The study shows that the same underlying mechanism, anaemia, is likely to be the cause of the adverse developmental effects both at high and low doses.

Critical period of sensitivity (Kawamura, 1993a; SBT-30-0044)

Single oral treatments of flumioxazin at 400 mg/kg bw were given to 5 pregnant rats/group on different gestational days (Kawamura, 1993; SBT-30-0044) in a non-GLP study performed observing EPA OPP 83-1 Guideline. Results showed that GD 12 is the day of greatest sensitivity to the developmental effects of flumioxazin including foetal death, reduced foetal bodyweight and VSD (see table below).

Table: *Summary results of the sensitive period-finding study with flumioxazin.*

Gestation day	Dose (mg/kg bw)	Embryonic Death (%)	Foetal body weight (g)		VSD (%)
			Male	Female	
11	400	2.7	3.34	3.22	6.9
12	**400**	**39.4**	**3.23**	**2.95**	**14.0**
13	400	16.1	3.73	3.49	5.8
14	400	9.9	3.59	3.14	4.7
15	400	6.3	3.67	3.46	2.2

A peak sensitive period was common to embryolethality, teratogenicity and growth retardation with flumioxazin. This suggests that the mechanism involved in all 3 endpoints is common. The effects were also similar to those reported after repeated dosage at 30 mg/kg bw/d. It also supports an identical mode of action leading to three types of developmental toxicities in the developmental toxicity study at 30 mg/kg bw/d and the sensitive period-finding study at 400 mg/kg bw.

Homogeneous maturation of erythroblasts (Ihara, 2011; SBT-0117)

In rats, haemopoiesis in yolk sac is characterised by a nearly simultaneous maturation of a relatively homogeneous population of erythroid cells. The morphology and population characteristics of these cells in rat embryos demonstrated that a vast majority of erythroblasts are polychromatophilic on gestational day 12, the day of the greatest sensitivity to flumioxazin, and orthochromatophilic erythroblasts on gestational day 14, when rat embryos were much less sensitive to the substance.

According to Ihara (2011), this simultaneous maturation might explain, in part, why flumioxazin induces a significant reduction in blood cells in rat embryos. In contrast to rats, a relatively heterogeneous population is observed in human primitive haemopoiesis where three types of erythroblast are found. It is conceivable that the type III erythroblast corresponds to the orthochromatophilic erythroblast only, whereas type I and type II correspond to less mature erythroblasts, presumably basophilic or polychromatophilic. The cell populations of type I, II, and III observed in embryonic yolk sac range from 7% to 40%, from 21% to 89%, and from 4% to 65%, respectively, from weeks 3-4 (start of the human primitive haemopoiesis) until week 8 (completion of ventricular septum formation). Therefore, it is argued that in humans, even if a particular population is lost due to flumioxazin toxicity, blood cell loss could not be as massive as in rats.

<u>Toxicokinetic considerations</u>

A physiologically based pharmacokinetic (PBPK) model for flumioxazin was developed in order to predict flumioxazin concentrations in the maternal blood and foetus of pregnant human. Flumioxazin concentrations in pregnant rats (orally dosed with 30 mg/kg bw/d) were used to develop the PBPK model in pregnant rats using physiological parameters from the literature and chemical-specific parameters from experimental results. An *in vitro* metabolism study using rat and human liver microsomes was conducted to analyse the species differences in the metabolism of flumioxazin between rat and human. In addition, a biliary excretion study was conducted in bile duct-cannulated female rats to determine the percentage of flumioxazin absorption after oral administration at 1000 mg/kg bw/d (Takaku, 2012a). Therefore, human specific factors were metabolic clearance and physiological parameters, and other chemical specific factors, such as absorption rate constant and partition coefficients to tissues, were assumed to be the same between humans and rats.

The developed human pregnant PBPK model demonstrated that flumioxazin concentration in the human foetus at oral dose of 1000 mg/kg bw/d would be 0.68 ppm (1.92 µM) (Figure 6) (Takaku,

2012a). This concentration is lower than the maximum no effect concentration of 5 µM in different human cells lines supporting the assumption that humans would not be susceptible to anaemia and thus to the developmental effects of flumioxazin.

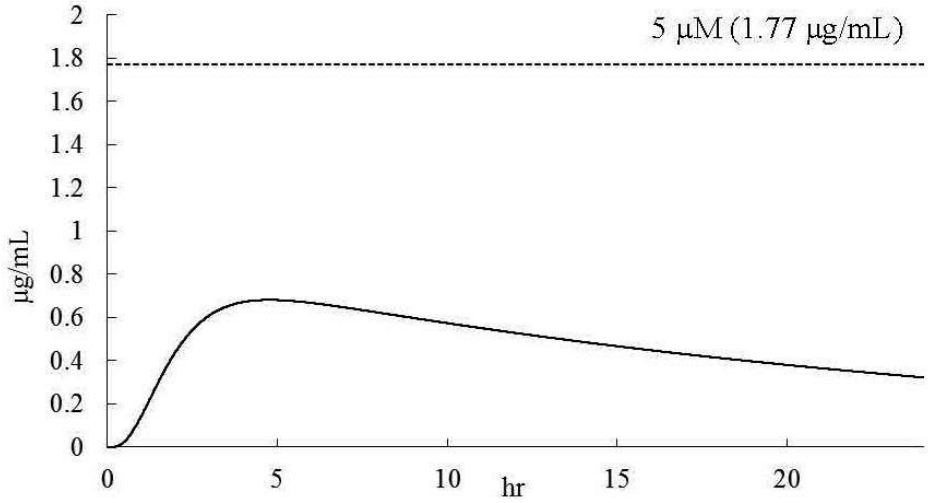


Figure 6: **Calculated flumioxazin concentration in the foetus of pregnant humans orally dosed with flumioxazin at a dose of 1000 mg/kg.**

RAC notes that human erythroblasts are concluded to be non-susceptible to flumioxazin when treated *in vitro* at concentrations as high as 5 µM and, that this concentration is expected to far exceed those attained in human embryos following flumioxazin exposure.

Human information: Flumioxazin and variegate porphyria (Meissner, 2014)

An expert opinion on human patients presenting with variegate porphyria in the context of potential adult or foetal anaemia and cardiac malformations, was prepared by Dr Peter Meissner, Professor and Head of Division of Medical Biochemistry Department of Clinical Laboratory Sciences, UCT Medical School, South Africa.

In humans, a 50% decrease in PPO activity is sufficient to result in the excessive production, and hence excretion of porphyrin intermediates from the haem pathway in many patients. This is highly variable and ranges from porphyrin excretion levels well within normal range, to grossly elevated concentrations. The loss of PPO activity appears not to be 'sensed' by the cell, and there is no compensatory PPO enzyme production or activity through other means. Assay of PPO activity in lymphocytes and other tissue derived from variegate porphyria patients show a loss of 50% of activity. About 40% of patients present with clinical symptomology.

By far the majority of patients with variegate porphyria are able to live a relatively normal lifestyle, and their life expectancy is normal. There are no reports of pregnancy-related problems in variegate porphyria mothers related to porphyria. To Dr Meissner's knowledge, there are no reports of the foetus presenting with symptoms of anaemia, nor cardiac malformation. Similarly children of variegate porphyria patients, themselves carrying (or not) the variegate porphyria gene do not present with specific symptoms of anaemia any more so than in a normal population. He is unaware of any higher incidence of anaemia in variegate porphyria children nor in adulthood. There are some families in his facility's database whose variegate porphyria status was monitored from birth to their mid-forties, themselves having produced children.

Dr Meissner concluded that in respect of the specific concerns surrounding possible foetal anaemia and cardiac malformation he is unaware of such symptomologies having been reported in variegate porphyria patients, or their children.

RAC notes that according to the literature, the vast majority of humans affected by variegate porphyria are heterozygous for the PPO gene (and therefore they have one intact allele for the PPO gene). There are only some reports on individuals with two mutated alleles of the PPO gene (compound heterozygotes). In compound heterozygotes patients, developmental abnormalities of the bone (short fingers) have been reported. RAC notes that maybe these effects on the bone in humans could be related to the effects seen in the rat foetuses i.e. increased incidence of wavy ribs and reduced ossification of sacrococcygeal vertebral bodies.

Other supporting studies

The background document Supplemental information summarises a wide array of additional *in vivo* studies conducted for clarifying the mode of action of flumioxazin. These studies include rat, mouse and rabbit pharmacokinetic studies examining placental transfer of flumioxazin, histopathological studies examining effects of flumioxazin on embryonic development, studies examining the mechanism of haematotoxicity in rats and studies of accumulation of PPIX in maternal liver and embryos of rats and rabbits.

Mechanism of action of developmental toxicity by flumioxazin

The integration of all pieces of available information allows the DS to propose a mode of action with the following key events:

- Flumioxazin inhibits PPO, which is the penultimate enzyme in haem biosynthesis and is localized in mitochondria (Figure 7A). Because of abnormal subcellular location, the resulting PPIX cannot be transformed to haem. Thus, inhibition of PPO in the rat embryo results in degeneration of foetal erythroblasts leading to anaemia (Figure 7B).
- Severe foetal anaemia leads to foetal death (Figure 7B).
- Surviving foetuses are growth-retarded as indicated by a decrease in body weight (Figure 12B). They compensate for this anaemia by pumping a greater volume of blood, which leads to observed enlargement of the heart just prior to closure of the interventricular foramen. These results in delayed closure of the foramen represented as VSD in the term foetus due to mechanical distortion of the heart or abnormal blood flow (Figure 7B).
- Concurrently serum protein is decreased in the foetus resulting in wavy ribs (Figure 7B).

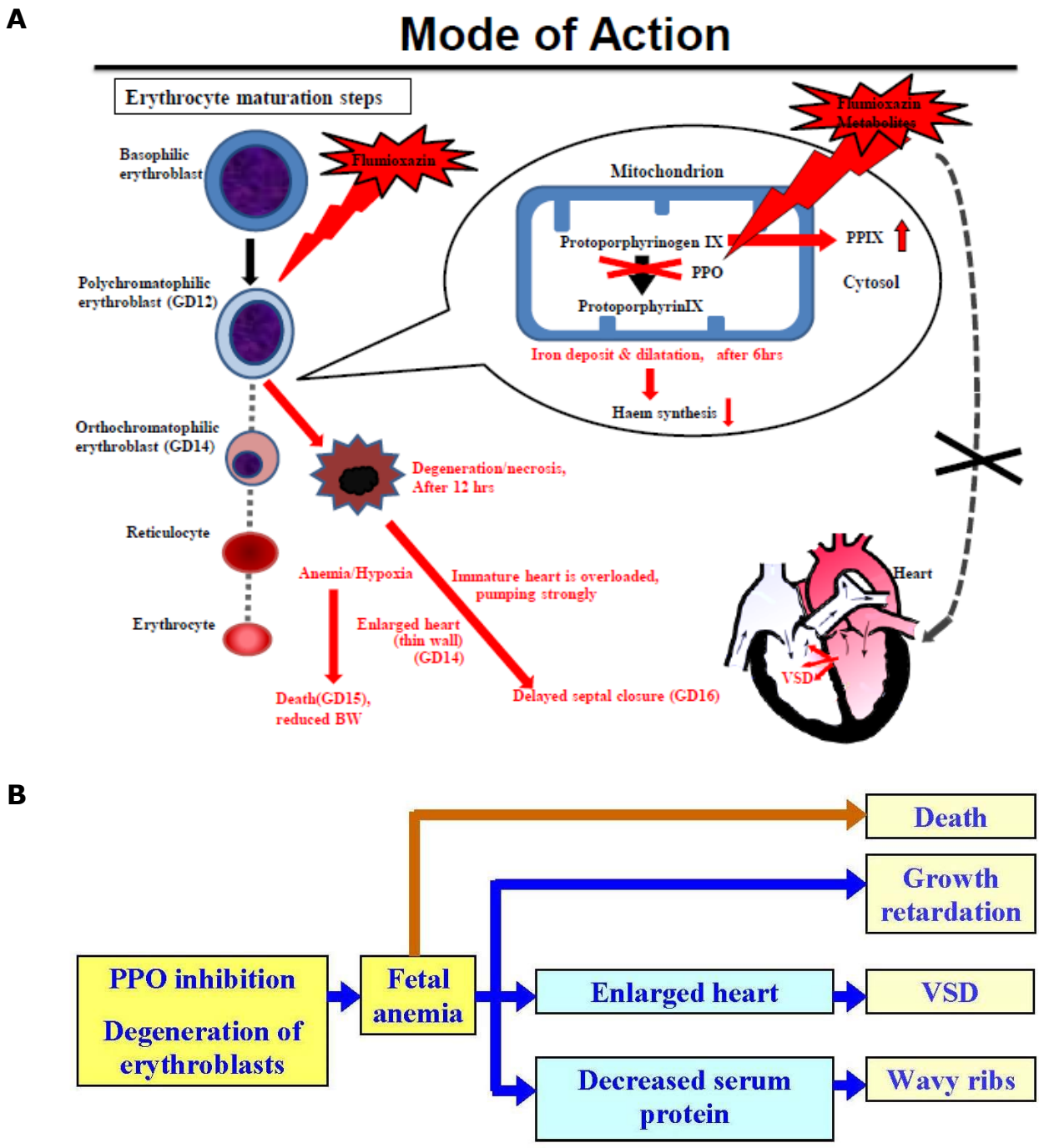


Figure 7: **Mechanism of developmental toxicity induced by flumioxazin.**

Human relevance of the proposed mode of action

The rat embryo is shown to be much more sensitive than the adult to the consequences of the induced anaemia. It was noted earlier that there is a species difference between rat and human in the erythropoiesis pattern during the developmental period. In the rat embryo, erythropoiesis begins with the production of a large, homogeneous wave of primitive erythroblasts over just a couple of days, which are released from the yolk sac into the circulation. This creates a very high demand for haem synthesis. GD 12, when the primitive cells are almost all in the form of polychromatophilic erythroblasts, is a critical period because, if these cells are damaged or killed, they cannot be replaced. In humans, the primitive stages of erythropoiesis in the yolk sac occur

over a much longer time period, which starts from the end of the second week of gestation and continues for several weeks until the foetal liver completely takes over production of red blood cells by 10-12 weeks of gestation. In contrast to the rat, erythropoiesis in humans produces a heterogeneous population of cells.

The *in vitro* studies investigating the effects of both flumioxazin and DHA in rat and human cell lines, which were induced to differentiate into erythroid cells, demonstrated a crucial and significant difference between the two compounds. These studies demonstrated convincingly that while there is no species difference with respect to inhibition of haem synthesis by DHA, there is a clear species difference for flumioxazin; human cells are insensitive to inhibition of haem synthesis by flumioxazin. The cell lines are appropriate models of human embryo erythroblasts (see Annex IV in background document for a detailed assessment of human relevance). The study with human $CD36^+$ cells is directly relevant, since these are physiologically close to primitive human erythroid cells. $CD36^+$ cells are foetal cells derived from cord blood, which is a rich source of haematopoietic progenitor cells. These cells can be viewed as closely related to yolk sac primitive erythroid cells as they can be differentiated into haem-synthesising cells under appropriate cell culture conditions. K562 cells are also a good model for human embryonic erythropoiesis. The K562 cell line exhibits phenotypic properties of embryonic erythroid progenitor cells and a quantitative increase in the expression of some of these properties can be achieved by differentiation induction. Thus, the phenotype expressed is more characteristic of early embryonic or foetal haematopoietic cells, as opposed to the adult phenotype.

It is highly unlikely that flumioxazin could cause anaemia in the embryo or developmental toxicity in humans given that human erythroid cell lines are shown to be insensitive to inhibition of haem synthesis by flumioxazin.

In summary, it is concluded that the mode of action in the rat is unlikely to be relevant to humans based on the following key factors:

- The species difference between rat and human in the erythropoiesis pattern during the critical developmental period.
- The insensitivity of erythroids derived from human K562 cells, $CD36^+$ cells and human induced pluripotent stem cells to inhibition of haem synthesis by flumioxazin at a concentration which equates approximately to a maternal *in vivo* dose of >1000 mg/kg bw/d.

Comparison with criteria

Fertility

RAC notes no treatment related effects on sexual function and fertility and in consequence supports the DS's proposal for **no classification of flumioxazin** as regards this hazard.

Development

RAC recognises that the induction of severe malformation such as VSD and foetal death in two well performed developmental toxicity studies in one species by two different routes of exposure might warrant classification within Category 1B (Category 1A cannot be considered since no human data is available).

Furthermore, RAC is of the opinion that no classification is not supported because the *in vitro* data clearly shows that flumioxazin has the potential to inhibit PPO in human foetuses and to lead to accumulation of PPIX. Moreover the IC_{50} of inhibition is in the same order of magnitude as in rats and therefore the accumulation of PPIX is likely in human foetuses at low exposure levels.

The Guidance on the Application of the CLP Criteria (July 2017) establishes that *"when there is mechanistic information that raises doubt about the relevance of the effect for humans, classification in Category 2 may be more appropriate"*

RAC notes that the DS used the principles of an established human relevance framework for non-cancer endpoints prepared by the International Programme on Chemical Safety (Boobis *et al.*, 2008). The framework describes a structured weight of evidence approach to assess the human relevance of a postulated mode of action in animals. The non-cancer human relevance framework requires 3 fundamental questions to be addressed in order to reach a conclusion on the human relevance of toxicological effects observed in animals:

1. Is the weight of evidence sufficient to establish a mode of action in animals?
2. Can human relevance of the mode of action be reasonably excluded on the basis of fundamental, qualitative differences in key events between experimental animals and humans?
3. Can human relevance of the mode of action be reasonably excluded on the basis of quantitative differences in either kinetic or dynamic factors between experimental animals and humans?

In the case of flumioxazin, RAC agrees with the DS that weight of evidence is sufficient to establish a single mode of action for developmental toxicity in the rat, which operates both at a high dose and at the lowest observed teratogenic dose.

RAC agrees with the DS that it cannot be concluded that there is a fundamental qualitative species difference in PPO inhibition between rat and human liver mitochondria, nor is there a fundamental difference between the adult rat and rat embryo/foetal tissues in sensitivity to PPO inhibition. However, for another key event, the inhibition of haem synthesis, there is a fundamental qualitative difference between rat and human erythroid cells to inhibition of haem synthesis by flumioxazin, whereby flumioxazin has no effect on haem synthesis in human erythroid cells, despite causing PPO inhibition in such cells.

Finally, RAC agrees with the DS that there is a marked kinetic difference between rats and humans. Although Boobis *et al.* (2008) noted that dismissing human relevance based on quantitative differences is likely to be infrequent, they mentioned that this is achievable where human exposure could not possibly be envisaged to reach the levels that would produce the toxicological effect. The pharmacokinetic modelling presented in the CLH report substantiates that it is unlikely that a plausible scenario whereby human exposure to flumioxazin could cause the developmental toxicity ascribed to the mode of action in the rat exists.

Overall, RAC considers that there is sufficient evidence to raise doubts about the relevance of the effect for humans and, on this basis, supports the DS's proposal to classify flumioxazin into **Category 2; H361d (Suspected of damaging the unborn child)**.

Additional references

Boobis, Doe, Heinrich-Hirsch, Meek, Munn, Ruchirawat, Schlatter, Seed, & Vickers, (2008. IPCS framework for analysing the relevance of a non-cancer mode of action for humans. Critical Reviews in Toxicology, 38; pp 87-96

Lavelle, .S., Schnatter, Travis, Swaen, Pallapies, Money, Priem, P. & Vrijhof,. 2012. Framework for integrating human and animal data in chemical risk assessment. Regulatory Toxicology & Pharmacology, 62; pp 302-312.

Ohls, 2011. Developmental Erythropoiesis. In: Fetal and Neonatal Physiology. Fourth Edition. Editors: Polin, R.A., Fox, W.W., Abman, S.H. Elsevier Saundres, Philadelphia USA. Pp1495-1519.

Takaku 2012a. Biliary excretion of [phenyl-U-14C]flumioxazin in female rats. Sumitomo Chemical Co., Ltd. Report No.: SBM-0092.

Takaku (2012b). Physiologically Based Pharmacokinetic Modeling of Flumioxazin in rats and humans. Sumitomo Chemical Co. Ltd. Report No.: SBM-0093.

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).