Committee for Risk Assessment

RAC

Annex 1

Background document

to the 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

The background document is a compilation of information considered relevant by the dossier submitter or by RAC for the proposed classification. It includes the proposal of the dossier submitter and the conclusion of RAC. It is based on the official CLH report submitted to public consultation. RAC has not changed the text of this CLH report but inserted text which is specifically marked as 'RAC evaluation'. Only the RAC text reflects the view of RAC.

Adopted

15 March 2019

CLH report

Proposal for Harmonised Classification and Labelling

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

Substance 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

Index Number: 613-166-00-x

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Dossier prepared by Sumitomo Chemical Co., Ltd in accordance with Article 37(6) of CLP.

ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON FLUMIOXAZIN (ISO); N-(7-FLUORO-3,4-DIHYDRO-3-OXO-4-PROP-2-YNYL-2H-1,4-BENZOXAZIN-6-YL)CYCLOHEX-1-ENE-1,2-DICARBOXIMIDE

CONTENTS

Part A

Part B

ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON FLUMIOXAZIN (ISO); N-(7-FLUORO-3,4-DIHYDRO-3-OXO-4-PROP-2-YNYL-2H-1,4-BENZOXAZIN-6-YL)CYCLOHEX-1-ENE-1,2-DICARBOXIMIDE

TABLES

Part A.

A short summary of Part A as it relates to the specific proposal for re-classification of flumioxazin for developmental toxicity is presented, rather than a duplication of the original Part A.

1 PROPOSAL FOR HARMONISED CLASSIFICATION AND LABELLING

1.1 Substance

Table 1: Substance identity

Substance 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
Annex VI Index number:	613-166-00-x
Degree of purity:	96.0% (w/w) (equivalent to 960 g/kg)
Impurities:	Confidential information. None of toxicological concern

1.2 Harmonised classification and labelling proposal

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 was included in Annex I of Commission Directive 2001/59/EEC adapting to technical progress for the 28th time Council Directive 67/548/EEC, 6th August 2001. The classification "Repr. Cat. 2; R61", corresponding to CLP classification "Repr. 1B; H360D", was based on developmental effects in the rat and presumed relevance to humans (refer to Part B, Section 4.11 for details). An extensive program of research with flumioxazin has successfully elucidated the mechanism of the developmental toxicity in rats and clarified the relevance to humans. The results of this research provide evidence that the rat is particularly sensitive to the toxic effects of flumioxazin whereas this is unlikely to be the case in humans. Therefore, a proposal to change the current harmonised classification and labelling 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 has been prepared. This proposal focuses on the change in classification 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 related to reproductive toxicity and therefore, this proposal only includes data relevant to the assessment of this hazard class.

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

	CLP Regulation
Current proposal for consideration by RAC	Re-classification of Repr. 1B H360D (May damage the unborn child) to Repr. 2 H361d (Suspected of damaging the unborn child)

1.3 Proposed harmonised classification and labelling based on CLP Regulation criteria

The proposed classification and labelling 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 based on the removal of the classification for reproductive toxicity is provided in Table 3.

Table 2: Proposed classification according to the CLP Regulation

CLP Annex I ref	Hazard class	Proposed classification	Proposed SCLs and/or M-factors	Current classification [1)]	Reason for no classification [2)]
3.7.	Reproductive toxicity	Repr. 2 H361d (Suspected of damaging the unborn child)	-	Repr. 1B (Hazard statement: H360D: May damage the unborn child)	Classified

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

Proposed labelling:

Signal word: Warning

Hazard pictogram: Repro Cat 2  Aquatic Acute Cat 1  Aquatic Chronic Cat 1

Hazard statements: H361d: Suspected of damaging the unborn child
H410: Very toxic to aquatic life with long lasting effects.

Proposed notes assigned to an entry:

Not applicable

2 BACKGROUND TO THE CLH PROPOSAL

2.1 History of the previous classification and labelling

Error! Reference source not found. was included in Annex I of Commission Directive 2001/59/EEC adapting to technical progress for the 28th time Council Directive 67/548/EEC, 6th August 2001. The classification "Category 2; R61 May cause damage to the unborn child", corresponding to CLP classification „Repr. 1B; H360D May damage the unborn child.", was based on effects observed in the rat developmental toxicity studies.

2.2 Short summary of the scientific justification for the CLH proposal

This new CLH report has been prepared for submission to the ECHA RAC in order to address the questions and concerns raised by the RAC in the meeting of 2-6 June 2014 (RAC 29) and

elaborated in the third draft Opinion Development Document (ODD) and the Opinion-to-Comments Table dated 30 April 2014. The RAC did not agree that non-relevance of the developmental toxicity findings to humans had been sufficiently demonstrated. On the other hand it was recognised that the CLP Regulation allowed the use of category 2 if there were doubts about the relevance of the mode of action (MoA) to humans. However, the RAC members concluded that the doubts in this case were not sufficient to warrant classification as Repr. 2. RAC therefore proposed to retain the current classification of Repr. 1B (H360D).

The critical RAC concerns focussed on the following aspects of the case for re-classification:

1. It was concluded that the MoA was plausible but not convincingly demonstrated (e.g. the MoA had been demonstrated at a high dose but had not been explored at a lower dose where developmental effects were also observed), while other mechanisms could not be excluded, based on the data presented.

2. It was further concluded that relevance for humans could not be excluded, although there may be quantitative differences between rats and humans.

The objective of this new CLH report is to address the concerns of the RAC. Therefore, only brief summaries of the studies already reviewed are presented. New studies have been conducted by the Notifier to clarify the MoA at the lowest dose level at which effects were observed in the regulatory developmental toxicity study in the rat, and to clarify the relevance of the MoA to humans. These studies are summarised in detail. Two additional studies submitted during the ECHA public consultation in October 2013 are also summarised in detail as only brief summaries of the results were presented previously. In addition, 2 documents are attached as Annex 1 and Annex 2. Those were prepared by the dossier submitter, focusing on MoA and human relevancy, and were provided to ECHA post RAC28 to address the experts' comments. This new CLH report discusses and summarises the key mechanistic work on mode of action and species differences in key events in Section 4.11.4 and in Section 4.11.6 assesses the weight of evidence against the CLP classification criteria, following the principles of an established human relevance framework.

The previous studies are considered to be adequate in assessing the developmental toxicity of flumioxazin, however since the initial inclusion of flumioxazin further mechanistic work has been undertaken to demonstrate the effects observed in the rat (embryolethality, teratogenicity (mainly ventricular septal defects and wavy ribs) and growth retardation) are species specific and unlikely to be relevant for humans.

Mechanistic research has established that toxic effects observed in the developmental studies and to a lesser extent the repeat dose studies (haematotoxicity) result from inhibition of the enzyme protoporphyrinogen oxidase (PPO)[1]. The effects reported in the rat developmental study were observed in the absence of maternal toxicity. 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. There is convincing evidence for a single mode of action causing the developmental toxicity in the rat. The sequence of key biological events in the proposed mode of action has been elucidated. Inhibition of PPO interferes with normal haem synthesis, which causes loss of blood cells leading to embryo-fetal anaemia, embryolethality and the development of malformations. Rats are particularly sensitive to the

[1] PPO is responsible for the 7th step in haem production, by removing hydrogen atoms from protoporphyrinogen IX to form protoporphyrin IX.

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. A new mechanistic developmental toxicity study has clearly demonstrated that the onset of fetal anaemia precedes the development of VSD at the lowest dose level at which such defects have been observed (30 mg/kg/day). This addresses the main concern of the 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. This new study also confirmed the finding of the previous developmental study that 30 mg/kg/day is the lowest observed effect level for a significant increase in the incidence of VSD.

In contrast, humans are unlikely to develop anaemia resulting from inhibition of PPO. This conclusion is based on;

(1) clinical findings that PPO deficient patients with Variegate Porphyria show no signs of anaemia. There are no reports of cardiac malformation in VP patients or their babies.

(2) experimental evidence that flumioxazin does not reduce haem synthesis in K562 cells and CD36+ cells, which are derived from human erythroleukemia and human cord blood, respectively

(3) experimental evidence that humans are less sensitive to PPO inhibition than rats.

(4) 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, 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. GD12, 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. Exposure to flumioxazin induces severe embryo anaemia and leads to 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 fetal liver completely takes over production of red blood cells by 10-12 weeks of gestation (Ohls, 2011). In contrast to the rat, erythropoiesis in humans produces a heterogeneous population of cells.

All the results of the new studies are consistent with those of the former studies previously submitted by the Notifier. The new studies demonstrate the reproducibility of the effects, confirm the MoA for the developmental effects and show that the MoA is unlikely to be of relevance for humans. The 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 humans. In clear contrast to DHA, flumioxazin does not inhibit haem synthesis *in vitro* in K562 cells or CD36+ cells. Moreover, human exposure levels are much lower than those attained in the toxicity studies.

Pharmacokinetic (PBPK) modelling in the rat and the human predicts that human erythroblasts would be insusceptible to flumioxazin at exposure equivalent to a maternal dose exceeding 1000 mg/kg/day 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.

Overall, it is concluded that the rat is an inappropriate model for assessing the developmental toxicity of flumioxazin in humans because, unlike humans, they are highly sensitive to PPO inhibition, resulting in embryo-fetal anaemia and consequent developmental toxicity. There is

unlikely to be a plausible 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.

According to the MoA and human relevance framework of both Boobis *et al* (2008) and Lavelle *et al* (2012), the MoA for reproductive toxicity is considered unlikely to be relevant to humans. Therefore, change of the current reproductive toxicity classification (Repr. 1B) is warranted and re-classification of flumioxazin as Repr.2. is considered justified based on the criteria for classification in Regulation EC 1272/2008.

2.3 Current harmonised classification and labelling

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

Classification

Repr. 1B, H360D (May damage the unborn child),

Aquatic Acute 1; H400,

Aquatic Chronic 1; H410,

M factor acute = 1000.
M factor chronic assigned by RAC29 = 1000

Labelling

Signal word: Danger

Hazard pictogram:
GHS08

GHS09

Hazard statements: H360D: May damage the unborn child

H410: Very toxic to aquatic life with long lasting effects

2.4 Current self-classification and labelling

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

Classification

As *per* the Annex VI entry

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 (MoA) for the developmental toxicity is not relevant for humans. However, RAC confirmed this classification in June 2014 for the following reasons:

- Although the proposed MoA leading to developmental toxicity was considered plausible by RAC, it was not convincingly explored at doses where developmental effects were in fact observed;
- Other MoA 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 MoA at low doses in developmental toxicity studies in rats and to clarify the relevance of the MoA to humans. This new CLH proposal (submitted by Czech Republic but prepared by the manufacturer) discussed and summarised the key mechanistic work on MoA and species differences in key events as well as assessed the weight of evidence against the CLP classification criteria.

3 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

There are data available to show that the existing harmonised classification for 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, Repr. 1B (H360D) in accordance with CLP is incorrect. Therefore, action is required at the Community level and this proposal seeks to amend the existing entry in Annex VI.

This proposal has been prepared by Sumitomo Chemical Co., Ltd. in accordance with Article 37(6) of CLP and submitted by the Czech Republic.

Part B.

As noted in the Introduction, this new CLH report focuses on addressing the concerns of the RAC from the RAC 29 meeting minutes and the accompanying documentation. Therefore, the scientific evaluation of the data is confined to the developmental toxicity of flumioxazin, particularly the new studies conducted by the Notifier.

SCIENTIFIC EVALUATION OF THE DATA

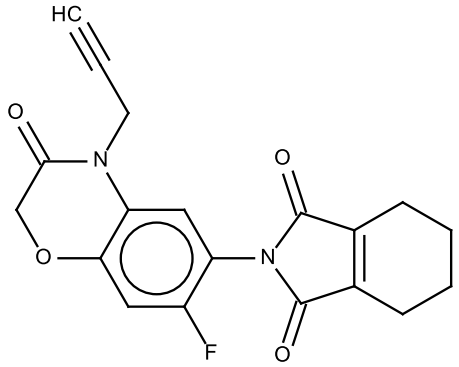
1 IDENTITY OF THE SUBSTANCE

1.1 Name and other identifiers of the substance

Table 3: Substance identity

EC number:	-
EC name:	-
CAS number (EC inventory):	103361-09-7
CAS number:	103361-09-7
CAS name:	2-[7-fluoro-3,4 –dihydro-3-oxo-4-(2-propynyl)-2H-1,4- benzoxazin-6-yl]-4,5,6,7-tetrahydro-1H-isoindole-1,3 (2H)- dione
IUPAC name:	*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
CLP Annex VI Index number:	613-166-00-x
Molecular formula:	$C_{19}H_{15}FN_2O_4$
Molecular weight range:	354.33

Structural formula:



1.2 Composition of the substance

Table 4: Constituents (non-confidential information)

Constituent	Minimum concentration	Concentration range	Remarks
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	96.0% (w/w) (equivalent to 960 g/kg)	96.0 - 100% (w/w)	

Current Annex VI entry: 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 Annex VI index number 613-166-00-x.

Details on the current classification are referred to in Part A, Section 2.3. There are M-factors associated with 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 and there are no notes associated with its Annex VI entry.

Table 5: Impurities (non-confidential information)

Impurity	Minimum concentration	Concentration range	Remarks
-	-	-	No impurities of toxicological concern

Current Annex VI entry: Not applicable

The manufacturer has requested that the impurity profile remains confidential, therefore this information is presented in the IUCLID 5 technical dossier only. The minimum purity of flumioxazin (ISO); *N*-(7-fluoro-3,4-dihydro-3-oxo-4-prop-2-ynyl-2*H*-1,4-benzoxazin-6-yl)cyclo hex-1-ene-1,2-dicarboximide is 96.0% and there are eight process impurities present. These have been taken into consideration in the classification and are not considered to be of additional concern.

Table 6: Additives (non-confidential information)

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

Current Annex VI entry: Not applicable

1.2.1. Composition of test material

Where available, the purity of the tested material is provided in the relevant sections. The reported studies are considered to be representative of the material as specified above.

1.3 Physico-chemical properties

This information was summarised previously and is not reproduced here.

2 MANUFACTURE AND USES

2.1 Manufacture

Flumioxazin is manufactured in Japan.

2.2 Identified uses

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 is a herbicide with a long history of agricultural use for the pre-emergence control of many annual broad-leaved weeds and some annual grasses.

3 CLASSIFICATION FOR PHYSICO-CHEMICAL PROPERTIES

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 is not classified with respect to physico-chemical properties. This is not considered further in this dossier.

4 HUMAN HEALTH HAZARD ASSESSMENT

As noted in the Introduction, only brief summaries of the studies already reviewed are presented. New studies have been conducted by the Notifier to clarify the MoA at the dose level where effects were observed in the regulatory developmental toxicity study in the rat, and to clarify the relevance of the MoA to humans. These studies are summarised in detail. Two additional studies submitted during the ECHA public consultation in October 2013 are also summarised in detail as only brief summaries of the results were presented previously.

The new studies are as follows:

Hosokawa, Y. (2015). Additional study to evaluate the potential of flumioxazin to cause foetal anaemia at developmentally toxic dose in rats. Sumitomo Chemical Co. Ltd., Report No.: SBT-0129

Abe, J. (2014). Inhibition of protoporphyrinogen oxidase activity by flumioxazin and its major metabolites, 3-OH flumioxazin, 4-OH flumioxazin and APF in human liver mitochondria. Sumitomo Chemical Co. Ltd., Report No.: SBT-0128

Kawamura, S. (2015a). Comparative effects of flumioxazin and dihydroartemisinin on the heme synthetic pathway and cell proliferation in rat erythroleukemia cells. Sumitomo Chemical Co. Ltd., Report No.: SBT-0132

Kawamura, S. (2015b). Comparative effects of flumioxazin and dihydroartemisinin on the heme synthetic pathway and cell proliferation in K562 cells. Sumitomo Chemical Co. Ltd., Report No.: SBT-0131

Kawamura, S. (2015c). Comparative effects of flumioxazin and dihydroartemisinin on the heme synthetic pathway and cell proliferation in human CD36+ cells. Sumitomo Chemical Co. Ltd., Report No.: SBT-0130.

Additional studies submitted during the ECHA public consultation in October 2013:

Kawamura, S. (2013b). Effects of flumioxazin on heme synthetic pathway and cell proliferation in rat erythroleukemia cells. Sumitomo Chemical Co. Ltd., Report No.: SBT-0125

Kawamura, S. (2013a). Effects of flumioxazin on heme synthetic pathway and cell proliferation in human CD36+ cells. Sumitomo Chemical Co. Ltd., Report No.: SBT-0126

The information provided in sections 4.7 and 4.10 are used only as supportive data for toxicity to reproduction.

4.1 Toxicokinetics (absorption, metabolism, distribution and elimination)

Refer to Section 4.11.4.2.

4.2 Acute toxicity

Not relevant for this proposal.

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

Not relevant for this proposal.

4.4 Irritation

Not relevant for this proposal.

4.5 Corrosivity

Not relevant for this proposal.

4.6 Sensitisation

Not relevant for this proposal.

4.7 Repeated dose toxicity

The results of the subchronic repeat dose toxicity studies considered relevant to this proposal are summarised. For convenience the chronic toxicity results in the combined chronic toxicity and carcinogenicity study in the rat are summarised in Section 4.10.

4.7.1 Non-human

4.7.1.1 Repeated dose toxicity: oral

The results of experimental studies on repeat dose after oral exposure in the rat are summarised in the following table. There was no evidence of anaemia in 90-day and 1-year oral toxicity studies in the dog up to the highest dose level of 1000 mg/kg/day and in a 28-day oral study in the mouse up to 10000 ppm, therefore these studies have not been summarised.

Table 7: Overview of experimental st udies on repeated dose toxicity after oral administration of flumioxazin

Method	Results	Remarks[1]	Reference
90d, rat (SD) (12 animals/sex/gp) oral: feed 0, 30, 300, 1000, 3000 ppm [equiv. 0, 2.3, 20.7, 69.7, 243.5 (M) and 0, 2.2, 21.7, 71.5, 229.6 mg/kg/d (F)] EPA OPP 82-1, GLP	NOAEL: *ca*. 20.7/21.7 mg/kg/d (male/female) LOAEL: ca. 69.7/71.5 mg/kg/d (m/f): mild anaemia and increased relative liver and kidney weights in males. At 244/230 mg/kg/d (m/f) there was significant and more marked anaemia with increased incidences of extramedullary haematopoiesis in the spleen and increased absolute spleen weights, relative liver and spleen weights in both males and females, and absolute thyroid weight and relative heart and thyroid weights in females, and relative kidney weights in males	1 (reliable without restriction) **key study** **purity: 98.4%**	Hagiwara (1989) SBT-91-0002
90d, rat (SD) (10 animals/sex/gp) oral: feed 0, 30, 300, 1000, 3000 ppm [equiv. 0, 1.9, 19.3, 65.0, 196.7 (M) and 0, 2.2, 22.4, 72.9, 218.4 mg/kg/d (F)] EPA OPP 82-1, GLP	NOAEL: *ca.* 19.3 mg/kg/d (male) LOAEL: *ca*. 65 mg/kg/d (male): anaemia and extramedullary haematopoiesis in spleen, increased liver, heart, kidney and thyroid weights NOAEL: *ca*. 2.2 mg/kg/d (female) LOAEL: *ca*. 22.4 mg/kg/d (female): anaemia and extramedullary haematopoiesis in the spleen At 197/218 mg/kg/d (m/f) there was more marked anaemia with increases in spleen, liver, heart and kidney weights in females, and increases in liver, heart, kidney and thyroid weights in males	1 (reliable without restriction) **key study** **purity: 94.8%**	Adachi (1991) SBT-10-0023

1. Studies evaluated according the criteria set out by Klimisch *et al* (1997)

90-day oral studies in rats

Two 90 day studies have been conducted in the rat using the same dose levels, one with technical grade flumioxazin (94.8%) and the other with a more pure grade (98.4 %). In both studies there were changes in the haematopoietic system indicative of anaemia. In the first study with 98.4% pure flumioxazin (Hagiwara, 1989 SBT-91-0002) decreased haemoglobin

concentration (Hb), decreased haematocrit, increased reticulocyte count, increased erythroblast count, and decreased red blood cell (RBC) count were observed at 3000 pm. Increased platelet count and decreased mean corpuscular haemoglobin (MCH) and mean corpuscular volume (MCV) were observed in the 1000 and 3000 ppm (both sexes) groups. In the second study with technical grade flumioxazin (Adachi, 1991 SBT-10-0023) haematology and bone marrow examination results indicated microcytic and hypochromic anaemia (decreased Hb concentration, decreased haematocrit, decreased MCHC, increased reticulocyte count, increased erythroblast count and decreased RBC count) at 1000 ppm and greater. Decreased MCV and MCH were also observed at 300ppm in females. The anaemia was associated with acceleration of the haematopoiesis, such as increased reticulocytes and erythroblasts in the blood, hypercellularity and decreased myeloid/erythroid ratio in the bone marrow. Extramedullary haematopoiesis in the liver and spleen were thought to be related and secondary effects to the anaemia. This was associated with increased spleen and liver weights in both studies. Pigmentation in the liver might have resulted from increased erythrocyte destruction and increases in heart weight might be regarded as compensatory hypertrophy resulting from lasting anaemia.

In the first study, the NOAEL was 300 ppm (20.7 mg/kg bw/d) based on changes in haematological parameters at 1000 ppm in both sexes, [along with increased incidences of extramedullary haematopoiesis in the spleen and increased absolute spleen weights, relative liver and spleen weights in both males and females, and absolute thyroid weight and relative heart and thyroid weights in females, and relative kidney weights in males at 3000 ppm]. In the second study the NOAEL was considered to be 30 ppm (2.2 mg/kg/day) based on haematological changes (including anaemia and extramedullary haematopoiesis) in females. For males, the NOAEL was considered to be 300 ppm (19.3 mg/kg/day) based on increased liver, heart, kidney, thyroid weights and evidence of low grade anaemia.

4.7.1.2 Repeated dose toxicity: inhalation

Not relevant for this proposal.

4.7.1.3 Repeated dose toxicity: dermal

The results of experimental studies on repeat dose after dermal exposure are summarised in the following table:

Table 8: Overview of experimental studies on repeated dose toxicity after dermal administration of flumioxazin

Method	Results	Remarks[1]	Reference
21d, rat (SD) (5 animals/sex/gp) dermal: semi-occluded 0, 100, 300, 1000 mg/kg/day EPA OPP 82-2, GLP	NOAEL: > 1000 mg/kg/day (male) based on no adverse effects observed up to the maximum dose tested NOAEL: ca. 300 mg/kg/day (female) based on decreased mean Hb and haematocrit values	1 (reliable without restriction) **key study** **purity: 94.8%**	Osheroff (1991) SBT-11-0026

1. Studies evaluated according the criteria set out by Klimisch *et al* (1997)

21-day dermal toxicity study (SBT-11-0026)

There were no indications of treatment related effects observed on body weight, food consumption, gross pathology, dermal response, clinical signs of toxicity or biochemical

parameters after dermal exposure to flumioxazin for 21 days. Signs of toxicity were limited to females in the high dose group with significant decreases in both Hb concentration and haematocrit values.

Based on the results of this study, the NOAEL was considered to be 1000 mg/kg/d for males (the maximum dose tested). For females the NOAEL was considered to be 300 mg/kg/d based on decreased mean Hb and haematocrit values.

4.7.1.4 Repeated dose toxicity: other routes

No relevant information.

4.7.1.5 Human information

No relevant information.

4.7.1.6 Other relevant information

None.

4.7.1.7 Summary and discussion of repeated dose toxicity

Overall, irrespective of the route of administration (oral (dietary) or dermal), toxic changes observed following flumioxazin exposure in the rat are associated with changes in the haematopoietic system. The changes are characteristic of anaemia and generally involve decreased Hb concentration, decreased haematocrit, decreased RBC count along with increases in reticulocytes and erythroblasts in the blood. Due to the decrease in the parameters mentioned, the compensatory mechanism involves hypercellularity and decreased myeloid : erythroid ratio in the bone marrow accompanied by acceleration of haematopoiesis, hence the increase in circulating immature RBC (i.e. reticulocytes and erythroblasts). Secondary effects to the anaemia involve extramedullary haematopoiesis in the liver and spleen, with evidence of increased erythrocyte destruction in the liver (manifest as pigmentation of the liver) and potential compensatory hypertrophy of the heart.

Note: Repeat dose toxicity studies showed that the rat was the most susceptible species and there was no evidence of anaemia in either the mouse (28-day, SBT-0014) or dog (90-day, SBT-0038 and 1 year, SBT-0039) studies (these data have not been reported in this proposal as they did not provide any evidence of toxicity relevant for the MoA proposed in the rat). However, the absence of haematological effects in these other species is discussed later in the context of species differences in the toxicity of flumioxazin.

Further discussion of the MoA of the anaemia is presented in Section 4.11.4.

4.8 Specific target organ toxicity (CLP Regulation) – Repeated exposure (STOT RE)

Not relevant for this proposal.

4.9 Mutagenicity (genotoxicity)

Not relevant for this proposal.

4.10 Carcinogenicity

4.10.1 Non-human information

4.10.1.1 Carcinogenicity: oral

Not relevant for this proposal. The results of the chronic toxicity component of the combined chronic toxicity and carcinogenicity study in the rat relevant to this proposal are summarised in the following table:

Table 9: Overview of experimental studies on chronic toxicity after oral administration of flumioxazin

Method	Results	Remarks[1]	Reference
52 or 104 wk, rat (SD) (50 animals/sex/gp) oral: feed 0, 50, 500, 1000 ppm [equiv. 0, 1.8, 18.0, 36.5 (M) and 0, 2.2, 21.8, 43.6 mg/kg/d (F)] EPA OPP 83-5, GLP	Non-neoplastic effects: NOAEL: *ca*. 1.8/2.2 mg/kg/d (male/female) (based on: chronic nephropathy (male) and haematological changes (anaemia, both genders)	1 (reliable without restriction) **key study** **purity: 94.8%**	Seki (1993) SBT-30-0040

1. Studies evaluated according the criteria set out by Klimisch *et al* (1997)

Summary of oral chronic toxicity data (SBT-30-0040)

Toxic changes observed were most prominently associated with changes in the haematopoietic system. Statistically significant haematological changes associated with anaemia were observed in male and female rats of the 500 and 1000 ppm groups. A slight increase ($p<0.05$) in extramedullary haematopoiesis in the spleen was observed in females of 1000 ppm group at week 53 and a significantly higher incidence was observed in males of the 500 and 1000 ppm groups ($p<0.01$) at week 105. The anaemia lasted throughout the treatment period; however it was not aplastic in nature or progressive. Chronic nephropathy was observed in males in the intermediate and high dose groups.

Based on the results of this study, flumioxazin was concluded not to be carcinogenic. The NOAEL for chronic toxicity was considered to be 50 ppm (equivalent to 1.8 and 2.2 mg/kg/d, for males and females respectively) based on chronic nephropathy (males) and haematological changes (anaemia, both genders).

Note: there was no evidence of adverse haematological effects in the mouse carcinogenicity study (SBT-30-0040). Therefore the study is not relevant to this proposal and it has not been summarised.

4.10.1.2 Carcinogenicity: inhalation

Not relevant for this proposal.

4.10.1.3 Carcinogenicity: dermal

Not relevant for this proposal.

4.10.2 Human information

None.

4.10.3 Other relevant information

None.

4.10.4 Overall summary and discussion of chronic toxicity data

Overall, chronic flumioxazin exposure *via* dietary administration in the rat was associated with increased incidences of extramedullary haematopoiesis and associated anaemia throughout the treatment period. The anaemia however was not aplastic in nature or progressive. No haematopoietic changes were observed in the mouse, suggesting that the rat was more susceptible. There were no treatment related oncogenic effects in either species.

Further discussion of the MoA of the anaemia is presented in section 4.11.4.2.

4.11 Reproductive Toxicity

4.11.1 Adverse effects on sexual function and fertility

The results of experimental fertility studies are summarised in the following table:

Table 10: Summary table of animal studies on adverse effects on sexual function and fertility

Method	Results	Remarks[1]	Reference
rat (SD) two-generation study (30 animals/sex/gp) oral: feed 0, 50, 100, 200, 300 ppm [equiv. P_1: 0, 3.2, 6.3, 12.7, 18.9 mg/kg/d (M) and 0, 3.8, 7.6, 15.1, 22.7 mg/kg/d (F); F_1: 0, 3.7, 7.5, 15.0, 22.4 mg/kg/d (M) and 0, 4.3, 8.5, 17.2, 25.6 mg/kg/d (F)] EPA OPP 83-4 , GLP	NOAEL parental toxicity: *ca*. 12.7 mg/kg/d (male/female) (based on adverse clinical signs, reductions in body weight, body weight gain, food consumption and organ weights) NOAEL offspring toxicity: *ca*. 7.5 mg/kg/d (male/female) (based on reduced pup body weights, increase in stillbirths with viability index and litter size reduced) NOAEL reproductive: *ca*. 15.1 mg/kg/d (female) (based on reduced gestation index in both P_1 and F_1 generations and an increase in the number of F_1 dams that did not deliver a litter)	1 (reliable without restriction) **key study** **purity: 94.8%**	Hoberman, A.M. (1992) SBT-21-0035

1. Studies evaluated according the criteria set out by Klimisch *et al* (1997)

There are no human data on adverse effects of flumioxazin on sexual function and fertility.

4.11.2 Short summary and overall relevance of the provided information on adverse effects on sexual function and fertility

Flumioxazin was administered via the diet to Sprague-Dawley rats for two successive generations, P1 and F1 to produce F1 and F2 litters, respectively.

Parental toxicity was evident from decreased body weights in F1 males accompanied by increased food consumption.

Litters in the 200 ppm group of the F2 generation had significant increases in stillbirths with related significant reductions in live born pups. Stillborns were not increased in the 300 ppm groups in the F2 generation, probably because there was a significantly increased incidence of embryo-fetal death earlier in gestation. Pup weight was significantly decreased in the pups of 200ppm group of the F1 generation. The gestation index (live litters delivered/pregnant females), number of pups delivered, number of liveborn pups, viability index, pup weight and litter size were significantly reduced in the 300ppm group in both generations. The reduction in the gestation index is related to the embryo-fetal death.

A general reduction in mating performance across all groups including controls was attributed to a genetically mediated problem in the rats supplied by Charles River breeding laboratory. No treatment-related effects on fertility were observed.

Based on the results of this study the NOAEL for parental toxicity was 200 ppm (12.7 mg/kg/d), based on clinical signs of toxicity and reductions in body weight, body weight gain, food consumption and organ weights. The reproductive NOAEL was also considered to be 200 ppm, based on reduced gestation index in both the P1 and F1 generations. The NOAEL for offspring toxicity was 100 ppm (7.6 mg/kg/d) based on reduced pup bodyweights, increase in stillbirths with viability index and litter size reduced and clinical and necropsy effects related to increased pup mortality.

4.11.3 Comparison with the CLP criteria

In the multi-generation study there was an increase in resorptions and a decrease in pup survival and average pup weight, which can be seen as an extension of the causal effects which resulted in the increased embryolethality and growth retardation observed in the rat developmental studies. No treatment related effects on sexual function and fertility were observed. Therefore, flumioxazin does not warrant classification for effects on sexual function and fertility. The reduction in the gestation index is attributed to the treatment related increase in embryo-fetal death.

4.11.4 Adverse effects on development

4.11.4.1 Standard studies

The results of rat and rabbit developmental studies are summarised in the following table:

Table 11: Summary table of animal studies on adverse effects on development

Method	Results	Remarks[1]	Reference
rat (SD) developmental study (22 females/gp) oral: gavage 0, 1, 3, 10, 30 mg/kg/d (exposed from GD 6 – 15) EPA OPP 83-3; GLP	NOAEL maternal toxicity: >30 mg/kg/day based on no signs of maternal toxicity observed up to the highest dose tested NOAEL developmental toxicity: *ca.* 10 mg/kg/day based on reduced number of live fetuses, reduced fetal body weights, increased incidence of cardiac ventricular septal defects, wavy ribs, curvature of the scapular and reduced ossification of sacrococcygeal vertebral bodies	1 (reliable without restriction) **key study** **purity: 94.8%**	Kawamura, S. (1990a) SBT-00-0012

Method	Results	Remarks[1]	Reference
rabbit (NZW) developmental study (20 females/gp) oral: gavage 0, 300, 1000, 3000 mg/kg/d (exposed GD 7 – 19) EPA OPP 83-3; GLP	NOAEL maternal toxicity: *ca*. 1000 mg/kg/day based on reductions in maternal body weight gains and relative and absolute food consumption NOAEL developmental toxicity: >3000 mg/kg/day based on no effects observed up to the highest dose tested	1 (reliable without restriction) **key study** **purity: 94.8%**	Hoberman (1991) SBT-11-0017
rat (SD) developmental study (24 females/gp) dermal: occluded (6 h/d) 0, 30, 100, 300 mg/kg/d (exposed from GD 6 – 15) EPA OPP 83-3; GLP	NOAEL maternal toxicity: >300 mg/kg/day based on no effects observed up to the highest dose tested NOAEL developmental toxicity: *ca*. 100 mg/kg/day based on reduced number of live fetuses, reduced fetal body weights, increased incidence of cardiac ventricular septal defects, wavy ribs and reduced ossification of sacrococcygeal vertebral bodies	1 (reliable without restriction) **key study** **purity: 94.8%**	Kawamura (1991) SBT-10-0021

1. Studies evaluated according the criteria set out by Klimisch *et al* (1997)

Rat oral developmental study (SBT-00-0012)

Flumioxazin was administered orally *via* gavage to groups of 22 pregnant female rats at concentrations of 0, 1, 3, 10 and 30 mg/kg/day from gestation day (GD) 6 to 15. Dams were culled on GD 20 and fetuses were removed by caesarean section and examined.

No signs of maternal toxicity were observed. The number of live fetuses and fetal body weights were significantly decreased in the high dose group, whereas the number of corpora lutea, implantations and sex ratio were similar in all groups.

The incidence of fetuses with cardiovascular abnormalities, primarily ventricular septal defects (VSD), was significantly increased in the 30 mg/kg/day group. Significantly increased incidences of scapular curvature (malformation) and wavy ribs (minor anomaly), were also observed in the high dose group. Treatment-related decreases in ossified sacrococcygeal vertebral bodies were considered to be related to the decreased fetal body weights, rather than due to a direct effect of the test material.

Based on the results of this study, the NOAEL for developmental toxicity was considered to be 10 mg/kg/day, based on increased incidence of mortality, cardiac VSDs, wavy ribs, curvature of the scapular and reduced ossification of sacrococcygeal vertebral bodies, and decreased body weight in the fetuses of rats. The maternal NOAEL was greater than 30 mg/kg/day.

Rabbit oral developmental study (SBT-11-0017)

Flumioxazin was administered orally *via* gavage to groups of 20 pregnant female rabbits at concentrations of 0, 300, 1000 and 3000 mg/kg/day from gestation day 7 to 19. Dams were culled on GD 29 and fetuses were removed by caesarean section and examined.

Reductions in maternal body weight gains and relative and absolute food consumption were observed in the 3000 mg/kg/day group.

There was no evidence of developmental effects and the NOAEL for developmental toxicity was considered to be greater than 3000 mg/kg/day (the highest dose tested). The maternal NOAEL was considered to be 1000 mg/kg/day based on reductions in maternal body weight gains and in relative and absolute food consumption.

Rat dermal developmental study (SBT-10-0021)

Flumioxazin was administered dermally to groups of 24 pregnant female rats at concentrations of 0, 30, 100 and 300 mg/kg/day from gestation day 6 to 15. Dams were culled on GD 20 and fetuses were removed by caesarean section and examined.

No signs of maternal toxicity were observed. The number of live fetuses and fetal body weights were significantly decreased in the high dose group, whereas the number of *corpora lutea* and implantations were similar in all groups.

The incidence of fetuses with cardiovascular abnormalities, primarily VSD was significantly increased in the 300 mg/kg/day group. Treatment related increases in wavy ribs (minor anomaly), and decreases in ossified sacrococcygeal vertebral bodies were also observed. The latter finding was considered to be related to the decreased fetal body weights, rather than due to a direct effect of the test material.

Based on the results of this study, the NOAEL for developmental toxicity was considered to be 100 mg/kg/d, based on increased incidence of VSD, wavy ribs and reduced ossification of sacrococcygeal vertebral bodies in the fetuses of rats, and decreased fetal body weights. The maternal NOAEL was greater than 300 mg/kg/day.

Human information

Specific human information relevant to the MoA of teratogenic effects in the rat is presented in Section 4.11.4.3.

4.11.4.2 Mechanistic studies

Mechanistic studies which were conducted after the publication of flumioxazin in the 28th ATP of the DSD and the previous mechanistic studies which established the MoA for teratogenicity in the rat are summarised below. These studies were evaluated in detail by the RAC and therefore, only brief summaries are presented. In addition, two studies submitted during the ECHA public consultation in October 2013 are summarised (SBT-0125 and SBT-0126).

Studies were conducted to establish the mode of teratogenic action in the rat and to establish relevance to humans.

Flumioxazin caused embryolethality, teratogenicity (mainly VSD and wavy ribs), and growth retardation in rats at 30 mg/kg without maternal toxicity but caused none of these effects in rabbits, even at a maternal toxic dose level of 3000 mg/kg (highest dose tested).

Based on the previous mechanism studies summarised in the Introduction and in Table 13 below, it is concluded that there is convincing evidence for a single MoA causing the developmental toxicity in the rat. The sequence of key biological events in the proposed MoA has been elucidated based on the studies summarised above. Inhibition of PPO interferes with normal haem synthesis, which causes loss of embryonic blood cells due to the targeted destruction of maturing erythroblasts in the yolk sac. The maturation of erythroblasts in the

yolk sac is a synchronised event, therefore catastrophic loss of this cell population leads to embryonic anaemia, embryolethality and development of malformations (VSD and wavy ribs). The critical period for sensitivity to the developmental effects of flumioxazin is day 12 of gestation and this correlates with the peak period of PPIX accumulation in the rat embryo resulting from the inhibition of PPO. Electron microscopy demonstrated that the target of flumioxazin is mitochondria in polychromatophilic erythroblasts, which is the main site for synthesizing haem, and subsequent degeneration of erythroblasts and erythrophagocytosis were observed.

The VSD caused by flumioxazin appears to result from inhibition of haem biosynthesis rather than from direct injury to embryonic heart tissue. Flumioxazin induced no abnormalities in cardiac muscle cells, but caused degeneration of mitochondria in polychromatophilic erythroblasts. Days 11 and 12 of gestation are critical periods of cardiac development in the rat when the interventricular septum begins to form (Marcela *et al*. 2012). [Note: gestation day is equivalent to day of embryonic development in Marcela *et al*. as day 0 of gestation was the day of observation of a vaginal plug in the flumioxazin studies.] Enlargement of the heart is a compensatory reaction to the embryo-fetal anaemia resulting from increased heart stroke volume observed in surviving fetuses. Enlargement of the heart precedes interventricular foramen closure, which occurs by day 16 of gestation in the normally developing embryo (Marcela *et al*. 2012), but is delayed in the flumioxazin exposed embryo. Therefore, the VSD is due to failure of heart closure resulting from mechanical distortion of the heart and/or abnormal blood flow rather than from direct toxic effects of flumioxazin on cardiac muscle tissue. In addition, decreased serum protein is observed in the fetus, presumably due to reduced production in the liver in response to hypoxia. The resulting osmotic imbalance causes oedema. Reduction of fetal serum protein leads to incomplete/delayed ossification of the ribs and the wavy ribs seen at term (Kast, 1994). Wavy ribs are induced in the later stages of rib chondrification and ossification, and are considered to be skeletal variations as opposed to malformations. Both minor delays in ossification and wavy ribs seem to be readily repairable via postnatal skeletal remodeling, are not mechanistically linked to malformation, and often are seen in the presence of maternal or fetal toxicity (Carney and Kimmel, 2007). The sensitivity to inhibition of PPO extracted from adult female livers is comparable with that of the fetus and it does not appear that there is a significant difference in sensitivity to the development of anaemia between adult and fetal rats. The MoA was explained in detail in the CLH report previously evaluated by the RAC.

In response to questions from the RAC 28 meeting and ODD, further clarification supporting the proposed MoA was provided by the Notifier, as summarised below (see Annex 1). In addition, an evaluation of other potential MoAs was also submitted and it was concluded that there is no compelling evidence for an alternative MoA for the developmental toxicity of flumioxazin (see Appendix 1 in Annex 1).

Observed types and incidences of developmental toxicity caused by flumioxazin are identical between repeated doses of 30 mg/kg and a single dose of 400 mg/kg. Considering there is a decrease in absorption rate with increment in oral dose, the difference in internal dose levels would not be as large as those calculated from dose levels orally administered. As a result of calculation with the PBPK model, Cmax and AUC of 400 mg/kg are 4 times and 6 times larger than that of 30 mg/kg. A single treatment at 400 mg/kg would be comparable to a repeated treatment at 30 mg/kg during the sensitive period. The evidence supports an identical MoA to be the basis for the developmental toxicity seen in both the developmental toxicity study at 30 mg/kg and the sensitive period-finding study at 400 mg/kg.

Rat embryos are susceptible to flumioxazin exposure within a shorter period of time than adults due to a single big wave of synchronous maturation of rat primitive erythroblasts in the yolk sac, while PPO inhibition is comparable between adults and embryos. In adults, the continuous haematopoiesis can compensate for the effects of flumioxazin over a short period of time and the cells no longer producing haem are non- or less sensitive to flumioxazin. Once the peak sensitive period has passed, during which almost all erythroblasts are polychromatophilic erythroblasts that synthesise haem actively (Wickrema et al., 1992), fetuses are less sensitive to flumioxazin since the major site of hematopoiesis shifts to the liver and continuing hematopoiesis occurs. Thus, the embryo would develop severe anaemia during the sensitive period, whereas the adult rat would not develop significant anaemia during several days of exposure to flumioxazin. A new study supporting the MoA was submitted during the ECHA consultation in October 2013 but was not summarized in detail (Kawamura 2013, SBT-0125). A more detailed study summary is provided below.

Subsequently, at the RAC 29 meeting it was concluded that the MoA was plausible but not convincingly demonstrated (e.g. the MoA had not been explored at the lowest dose at which effects were observed) while other mechanisms could not be excluded, based on the data presented. In response to this concern the Notifier conducted a new developmental toxicity study at dose levels including 30 mg/kg/day to demonstrate fetal anaemia at the teratogenic dose level in the regulatory study (see summary in Section 4.11.4.3.2).

4.11.4.2.1 *In vitro*

Table 13: Overview of previous *in vitro* MOA studies conducted on flumioxazin

Method	Results	Remarks[1]	Reference
Haematotoxicity studies			
effects of flumioxazin on haem synthetic pathway and cell proliferation in rat erythroleukemia cells No guideline available; non-GLP	Results demonstrate the sensitivity of the rat erythroid cell to PPIX accumulation and reduced haem synthesis. No evidence of cell proliferation	2 (reliable with restrictions) **Flumioxazin** **key study** **purity: not stated**	Kawamura (2013b) SBT-0125
PPO and PPIX mechanistic studies on flumioxazin and its metabolites			
inhibitory action against the enzyme PPO obtained from rat liver mitochondria, *in vitro* S-53482: 10 pM - 1 µM 3-OH S-53482: 100 pM - 10 µM 4-OH S-53482: 1 nM - 100 µM APF: 1 nM - 100 µM No guideline available; non-GLP	Flumioxazin has the strongest inhibitory activity among the 4 substances tested, followed by 3-OH S-53482 and 4-OH S-54382, which were 13.7 and 147 times weaker than flumioxazin. APF does not have any inhibitory activity against PPO up to 100 µM.	2 (reliable with restrictions) **key study** **Flumioxazin purity: 99.4%** **3OH S-53482 purity: 99.6%** **4OH S-53482 purity: 99.0%** **APF purity: 99.9%**	Abe, J. (2011a) SBT-0118

Method	Results	Remarks[1]	Reference
SB herbicides on PPO activity in rat and rabbit liver mitochondria	All three SB series herbicides inhibited mammalian PPO activity. The IC50 values for S-53482 (flumioxazin), S-23121 & S-23031 were respectively 23, 36 and 2230 nM for rats and 300, 690 and 12500nM for rabbits. The relative sensitivity of the species to PPO inhibition by SB series herbicides was rat > rabbit.	2 (reliable with restrictions) **key study** **S-53482 purity: 94.8%** **S-23121 purity: 94.7%** **S-23031 purity: 94.7%**	Noda (1995) SBT-0058
PPO activity in rat and rabbit tissue	Adult liver and embryo mitochondria showed similar sensitivity to PPO inhibition by the test compounds, S-53482 (flumioxazin), S-23121 and S-23031,with the rabbit enzyme results showing less sensitivity to inhibition by the test compounds than the rat enzyme. The relative potency for inhibition was flumioxazin (S-53482) > S-23121 > S-23031	2 (reliable with restrictions) **key study** **S-53482 purity: 94.8%** **S-23121 purity: 94.7%** **S-23031 purity: 94.7%**	Green & Dabbs (1993) SBT-31-0045

Study examining effects of flumioxazin on the haem synthetic pathway and cell proliferation in rat erythroleukemia cells (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). The REL cell line was derived from transplantable tumors from 7,12 dimethylbenz (a) anthracene-induced erythroleukemia in the Long-Evans rat. 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.

A 50 mM flumioxazin solution was diluted from the stock solution with DMSO, and mixed with HMBA medium to prepare 5.0 μM flumioxazin medium, which was diluted by adding HMBA medium containing 0.01 % DMSO to make test medium containing 1.0 μM, 0.3 μM, 0.1 μM and 0.01 μM flumioxazin. HMBA medium containing 0.01 % DMSO was used as the control.

REL cells were centrifuged at 1000 rpm for 5 minutes, re-suspended in each flumioxazin medium (5.0 μM, 1.0 μM, 0.3 μM, 0.1 μM, 0.01 μM and 0 μM), plated onto 60 mm dishes at 5×10^5 cells/ 5 mL/ dish, and were placed in a CO_2 incubator. The seeding day is defined as Day 0. Control cell suspension at Day 0 was analyzed for haem and PPIX content. On Days 2, 4, 6 and 8, the plated cells were centrifuged at 1000 rpm for 5 minutes, washed with PBS(-) and re-suspended to measure the cell numbers. After that, the cells were centrifuged again, and stored at -80 ℃ until measurement. On Days 2, 4, and 6, the cells were subcultured at a concentration of 1×10^5cells/ mL in new dishes at 5 mL/ dish. Extraction and analysis of PPIX and haem were conducted according to previously described methods.

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. These results are in contrast to the study of PPIX accumulation and haem synthesis in human K562 cells in which PPIX accumulation was observed but with no inhibition of haem synthesis (Kawamura 2012, SBT-0119)).

The maximum accumulations of PPIX and maximum inhibition of haem synthesis are summarised in Table 14.

Table 12: Mean accumulation of PPIX and inhibition of haem synthesis in REL cells

Concentration of 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]

n = 4 per group

Table 13: Overview of previous mechanistic *in vitro* studies to establish human relevance

Method	Results	Remarks[1]	Reference
Species comparison studies of PPO inhibition and PPIX accumulation (refer to studies on rat and rabbit in Section 4.11.4.2.2)			
species difference in accumulation of PPIX in primary hepatocytes from rat, rabbit, monkey & human, *in vitro* 0.01 - 0.3 µg/mL No guideline available; non-GLP	The induction ratios of PPIX following treatment with flumioxazin at 0.3 µg/mL were 10.3, 1.1, 1.4 and 4.4-fold in primary hepatocytes of rat, rabbit, monkey and human respectively. These results suggest that rat hepatocytes are more sensitive to flumioxazin treatment than the other 3 species, including human.	2 (reliable with restrictions) **Flumioxazin** **key study** **purity: 99.4%**	Abe, J. (2011b) SBT-0120
Inhibition of PPO by flumioxazin in rat, human and rabbit liver	The IC_{50} values for flumioxazin after a 20 min incubation period for the inhibition of PPO activity in liver from rats, rabbits and humans were 0.00715 ± 0.0021 µM; 0.138 ± 0.0739 µM and 0.0173 ± 0.0044 µM, respectively. The relative sensitivity of the species to PPO inhibition by flumioxazin was rat > human > rabbit.	2 (reliable with restrictions) **Flumioxazin** **key study** **purity: 94.8%**	Green & Dabbs (1996) SBT-0060
Mechanistic studies using human cell lines			

Method	Results	Remarks[1]	Reference
effects of flumioxazin on haem synthetic pathway and cell proliferation in human CD36+ cells No guideline available; non-GLP	PPIX accumulation in CD36+ cells was observed at concentrations of 1 µM and greater in dose-dependent manner, however there was no effect on cell proliferation or haem synthesis at the highest dose tested (5 µM).	2 (reliable with restrictions) **Flumioxazin** **key study** **purity: not stated**	Kawamura (2013a) SBT-0126
K562 cell differentiation into erythroid cells in the presence of flumioxazin, *in vitro* 0.01 - 5 µM No guideline available; non-GLP	PPIX accumulation in K562 cells was observed at concentrations of 1 µM and greater in dose-dependent manner, however there was no effect on cell proliferation or haem synthesis at the highest dose tested.	2 (reliable with restrictions) **Flumioxazin** **key study** **purity: not stated**	Kawamura, S. (2012a) SBT-0119
K562 cell differentiation into erythroid cells in the presence of metabolites of flumioxazin, *in vitro* 5 µM No guideline available; non-GLP	There was no effect on PPIX content, haem synthesis or cell proliferation when K562 cells were treated with the metabolites, while flumioxazin increased PPIX in K562 cells.	2 (reliable with restrictions) **Flumioxazin metabolites** **key study** **purity: not stated**	Kawamura, S. (2012b) SBT-0123
PBPK modelling			
PBPK modelling of flumioxazin in rats and humans, *in vitro* and *in silico* 5.6 – 100 µM No guideline available; non-GLP	The developed human PBPK model demonstrated that the human fetal exposure to flumioxazin following a maternal oral dose of 1000 mg/kg would be 0.68 ppm (1.92 µM), showing that exposure to flumioxazin in a human fetus would be relatively low, even following a maternal dose of 1000 mg/kg.	2 (reliable with restrictions) **key study** **[phenyl-U ^{14}C]flumioxazin** **purity: 98.6%**	Takaku, T. (2012b) SBM-0093

1. Studies evaluated according the criteria set out by Klimisch *et al* (1997)

A new study of the effects of flumioxazin on the haem synthetic pathway and cell proliferation in human CD36+ cells was submitted but was not summarized in detail (Kawamura (2013a) SBT-0126). A more detailed study summary is provided below.

Study examining effects of flumioxazin on the haem synthetic pathway and cell proliferation in human CD36+ cells (SBT-0126)

Human CD36+ cells were derived from human CD34+ cells in culture. Human CD34+ cells were positively isolated from human cord blood using a direct immunomagnetic CD34 MicroBead labeling system and the CD36+ cells were positively isolated using a direct immunomagnetic CD36 MicroBead labelling system. 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. Concentrations of haem and protoporphyrin IX (PPIX) were determined.

Hematopoietic Progenitor Growth Medium (HPGM) containing 25 ng/mL SCF, 3 U/mL EPO, 10 ng/mL IL-3 and 10 ng/mL IL-6 and 1 % P/S was used as base medium. 50 mM flumioxazin

solution was diluted from a stock solution with DMSO, and mixed with base medium to prepare 5.0 µM flumioxazin medium which was diluted by adding base medium containing 0.01 % DMSO to make test medium containing 1.0 µM, 0.1 µM and 0.01 µM flumioxazin. Base medium containing 0.01 % DMSO was used as the control.

Human CD36+ cells were centrifuged at 1000 rpm for 5 minutes, re-suspended in each flumioxazin medium (5.0 µM, 1.0 µM, 0.1 µM, 0.01 µM and 0 µM), plated onto 30 mm dishes at 6×10^5 cells/ 3 mL/ dish , and were placed in a CO_2 incubator. The seeding day is defined as Day 0. Control cell suspension was analyzed for haem and PPIX content. On days 2, 4, 6 and 8, the plated cells were centrifuged at 1000 rpm for 5 minutes, washed with PBS(-) and re-suspended to measure the cell numbers. After that, the cells were centrifuged again, and stored at -80 ℃. On days 2, 4, and 6, the cells were subcultured at a concentration of 2×10^5cells/ mL in new dishes at 3 mL/ dish. Extraction and analysis of PPIX and haem were conducted according to previously described methods.

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. This is consistent with the results of a similar study on human K562 cells (Kawamura 2012, SBT-0119).

The maximum accumulations of PPIX on Day 8 and the corresponding data on haem synthesis are summarised in Table 16.

Table 14: Mean accumulation of PPIX and results of haem synthesis in CD36+ cells

Concentration of 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]

n = 4 per group

4.11.4.2.2 *In vivo*

Table 15: Overview of previous *in vivo* MOA studies conducted on flumioxazin

Method	Results	Remarks[1]	Reference
Pharmacokinetic and placental transfer studies			
rat pharmacokinetic study (3 females/gp) oral: gavage rat (Crl:CD (SD)): 1000 mg/kg/ 3.7 MBq/single dose No guideline available; non-GLP	The total amounts of ^{14}C excreted into bile and urine and ^{14}C remaining in the carcass showed that the absorption (bile + urine + carcass) in females was 12.3% after a single oral administration of flumioxazin at 1000 mg/kg.	2 (reliable with restrictions) **key study** **[phenyl-U ^{14}C]flumioxazin purity: 98.6%** **Flumioxazin**	Takaku, T. (2012a) SBM-0092

Method	Results	Remarks[1]	Reference
rat / rabbit pharmacokinetic study (4 females/gp) oral: gavage rat (HW): 30 mg/11.3 MBq/5 mL/kg/d rabbit (NZW): 30 mg/1.12 MBq/0.5 mL/kg/d (exposure from GD 6 – 12) Japanese guidelines on non-clinical pharmacokinetic studies no.496; non-GLP	In rats significantly higher transfer of radioactivity to blood cells was observed compared with rabbits. Elimination of radioactivity from female reproductive tissue of both species was slower than that from plasma, with only a small amount of radioactivity being transferred to the fetus.	2 (reliable with restrictions) **key study** **[phenyl-U ^{14}C]flumioxazin purity: 99.9%** **Flumioxazin purity: 99.4%**	Shirai, N. (2009) SBM-0081
rat / rabbit (SD / JW) study examining placental transfer of flumioxazin oral: gavage 30 mg/kg (exposure: rats GD 12 / rabbits GD10) EPA OPP 85-1, non-GLP	In rats significantly higher transfer of radioactivity to blood cells was observed compared with rabbits. Elimination of radioactivity from female reproductive tissue of both species reached maxima 2-4 hrs after administration and decreased rapidly thereafter, with only a small amount of radioactivity being transferred to the fetus.	2 (reliable without restriction) **key study** **[phenyl-U ^{14}C]-flumioxazin** **purity: >99%**	Isobe (1993) SBM-30-0032
rat / mouse (SD / ICR) study examining placental transfer of flumioxazin (24 animals/gp) oral: gavage 30 mg/kg (exposure:GD12) EPA OPP 85-1, non-GLP	In mice significantly higher transfer of radioactivity to blood cells was observed compared with rats. Elimination of radioactivity from female reproductive tissue of both species was slower than that from blood (blood cell & plasma), with only a small amount of radioactivity being transferred to the fetus.	1 (reliable without restriction) **key study** **[phenyl-U ^{14}C]flumioxazin** **purity: >99%**	Isobe (1992) SBM-20-0015
Further developmental mechanistic studies			
rat (SD) study examining the critical period for developmental toxicity (5 females/gp) oral: gavage 400 mg/kg (exposure: single dose on GD 11, 12, 13, 14 or 15) EPA OPP 83-1, non-GLP	The data confirmed that the most sensitive developmental stage common to VSD, embryonic mortality and reduced fetal body weight was GD 12. Since all 3 endpoints peaked in incidence in the group treated on GD 12, it is suggested that the mechanism involved in all 3 endpoints is common to teratogenicity, embryolethality and growth retardation.	2 (reliable with restrictions) **key study** **Flumioxazin** **purity: 94.8%**	Kawamura (1993a) SBT-30-0044

Method	Results	Remarks[1]	Reference
rat / rabbit (SD / JW) study examining the histopathological effects of flumioxazin on embryonic development oral: gavage 0, 1000 mg/kg (single exposure GD 12) EPA OPP 83-3; non-GLP	Histopathological changes were restricted to rat embryos only. Microscopy demonstrated mitochondrial lesions including abnormal iron deposition in polychromatophilic erythroblasts and subsequent degeneration of erythroblasts and erythrophagocytosis. Histological changes in the heart, such as thinning ventricular walls followed the erythroblastic lesion. It is concluded that flumioxazin induced VSD by altering haematological function *via* the inhibition of haem synthesis rather than producing a directly injurious effect on the heart. The embryo compensates for the anaemic hypoxia by increasing heart stroke volume, leading to hypertrophy of the heart. VSD defects result from mechanical distortion of the heart.	2 (reliable with restrictions) **key study** **Flumioxazin** **purity: 94.8%**	Kawamura & Yoshioka (1997) SBT-0064 and Kawamura (1993b) SBT-30-0043
rat (SD) study examining the pathogenesis of developmental effects produced by flumioxazin oral: gavage 0, 400 mg/kg (single exposure GD 12) EPA OPP 83-3; non-GLP	Data from this study suggest that the enlarged heart, oedema and anaemia preceding the occurrence of fetal mortality may be instrumental in the cause of death. Similarly, the occurrence of enlarged heart preceding the failure of the interventricular closure would be related to the pathogenesis of this finding.	2 (reliable with restrictions) **key study** **Flumioxazin** **purity: 94.8%**	Kawamura (1997) SBT-0065
rat (SD) developmental study (21 females/gp) oral: gavage 0, 1, 3, 10, 20 mg/kg/d (exposed GD 6 – 15) EPA OPP 83-3; GLP	NOAEL maternal toxicity: >20 mg/kg/day based on no signs of maternal toxicity observed up to the highest dose tested NOAEL developmental toxicity: *ca*. 10 mg/kg/day based on increased incidence ofVSD, growth retardation and embryolethality	1 (reliable without restriction) **key study** **Flumioxazin anologue** **S-23121** **purity: 94.7%**	Kawamura (1990b) PPT-00-0023
rabbit (NZW) developmental study (20 females/group) oral: gavage 0, 2, 4, 8, 15 mg/kg/d (exposed GD 7 – 19) EPA OPP 83-3; GLP	NOAEL maternal toxicity: *ca*. 2 mg/kg/day based on reductions in maternal body weight / body weight gain and relative / absolute food consumption NOAEL developmental toxicity: >15mg/kg/day based no effects observed up to the highest dose tested	1 (reliable without restriction) **key study** **Flumioxazin anologue** **S-23121** **purity: 94.7%**	Hoberman (1990) PPT-01-0020
rat (SD) developmental study (25 females/group) oral: gavage 0, 50, 500, 1500 mg/kg/d (exposed GD 6 – 15) EPA OPP 83-3; GLP	NOAEL maternal toxicity: >1500 mg/kg/day based on no signs of maternal toxicity observed NOAEL developmental toxicity: >1500mg/kg/day based on no effects observed up to the highest dose tested	1 (reliable without restriction) **key study** **Flumioxazin anologue** **S-23031** **purity: 94.4%**	Lemen (1991a) SAT-11-0024

Method	Results	Remarks[1]	Reference
rabbit (NZW) developmental study (17 females/group) oral: gavage 0, 100, 200, 400, 800 mg/kg/d (exposed GD 7 – 19) EPA OPP 83-3; GLP	NOAEL maternal toxicity: *ca*. 400 mg/kg/day based on reductions in maternal body weight / body weight gain and mortality NOAEL developmental toxicity: >800mg/kg/day based on no effects observed up to the highest dose tested	1 (reliable without restriction) **Flumioxazin anologue** **key study** **S-23031** **purity: 94.4%**	Lemen (1991b) SAT-11-0025
Haematotoxicity studies			
development of rat erythroblasts in rat embryos, *ex vivo* rat (SD) male/female No test material added, study used to examine differentiation of developing erythrocytes No guideline available; non-GLP	In rats differentiation of circulating erythroblasts in rat embryos from embryonic day 11 to 14 was synchronised.	2 (reliable with restrictions) **key study** **purity: n/a**	Ihara, R. (2011) SBT-0117
rat (SD) study examining the mechanism of haematotoxicity (up to 30 animals/sex/gp) oral: feed study 1: 0, 3000, 10000 ppm study 2: 0, 3000 ppm (exposure: study 1: 37 days; study 2: 15 days) No guideline available; non-GLP	Flumioxazin induced anaemia in rats can be classified as sideroblastic anaemia resulting primarily from the defective haem pathway during the process of haemoglobin biosynthesis considering the increase in porphyrins and siderocytes. The increased blood porphyrin level suggested the S-53482 induces porphyria in rats.	2 (reliable with restrictions) **Flumioxazin** **key study** **purity: 94.8%**	Yoshida (1996) SBT-0059

Method	Results	Remarks[1]	Reference
PPO and PPIX mechanistic studies on flumioxazin and its metabolites			
rat / rabbit (SD / JW) PPIX accumulation in maternal liver and embryos post single administration (up to 4 females/gp) oral: gavage rat / rabbit: 1000 mg/kg (exposure: single dose on GD 12) EPA OPP 83-3;non-GLP	PPIX accumulated in rat embryos up to 12 h post dosing, reaching 200-fold greater than the control values. In contrast PPIX levels in rabbits remained very low throughout the post dosing period. The species difference in PPIX accumulation in embryos correlates with that of the developmental toxicity produced by flumioxazin.	2 (reliable with restrictions) **Flumioxazin** **key study** **purity: 94.8%**	Kawamura (1996a) SBT-0061 and Kawamura (1993c) SBT-30-0042
rat (SD) PPIX accumulation in maternal liver and embryos post single administration (up to 5 females/gp) oral: gavage rat: 1000 mg/kg (exposure: single dose on GD 12) EPA OPP 83-3;non-GLP	PPIX accumulated in both whole embryos and maternal livers following administration of flumioxazin and S-23121. The extent of accumulation in embryos was greater than that observed in maternal livers, with the increase of PPIX in the embryos up to 290-fold greater than the control value. For S-23031, PPIX accumulation was not observed in either rat embryo or maternal liver samples.	2 (reliable with restrictions) **key study** **flumioxazin purity: 94.8%** **S-23031 purity: not stated** **S-23212 purity: not stated**	Kawamura (1996b) SBT-0062 and Kawamura (1993d) SBT-30-0042
rat / rabbit (SD / JW) PPIX accumulation in maternal liver and embryos (up to 5 females/gp) oral: gavage rat / rabbit: 400 / 1000 mg/kg (exposure: single dose on GD 10 - 15) EPA OPP 83-3;non-GLP	PPIX accumulated in whole embryos of rats, peaking on GD 11 to 12. Accumulation of PPIX was not observed in maternal rat or rabbit livers or in rabbit embryos.	2 (reliable with restrictions) **Flumioxazin** **key study** **purity: 94.8%**	Kawamura (1996c) SBT-0063 and Kawamura (1993e) SBT-30-0042

The studies presented in Section 4.11.4.3 show that 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.

In contrast, humans are unlikely to develop anaemia from PPO inhibition. This conclusion is based on:

1. Clinical findings that PPO deficient patients with Variegate Porphyria (VP) show no signs of anaemia. There are no reports of cardiac malformations in VP patients or their babies.

2. Experimental evidence that flumioxazin and its metabolites do not reduce haem production in human K562 cells, which are derived from human erythroleukemia, nor in human CD36+ cells, which are derived from human cord blood

3. Experimental evidence that humans are less sensitive to PPO inhibition than rats.

4. 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, 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. GD12, 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. Exposure to flumioxazin induces severe embryo anaemia and leads to 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 fetal liver completely takes over production of red blood cells by 10-12 weeks of gestation (Ohls, 2011). In contrast to the rat, erythropoiesis in humans produces a heterogeneous population of cells.

PBPK modelling in the rat and the human predicts that human erythroblasts would be insusceptible to flumioxazin at an exposure equivalent to a maternal dose exceeding 1000 mg/kg/day, thus demonstrating the large species difference in sensitivity. 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.

In addition, a recent medical surveillance report conducted on manufacturing plant personnel (Nishioka, 2011 SBT-0116) revealed no evidence of haematotoxicity, or other adverse health effects in workers (n=15) who have been involved in the manufacture of flumioxazin for the last decade. This is considered to demonstrate not only effective use of personal protective equipment, but also the intrinsic low toxicity of flumioxazin.

Overall, it was concluded that the rat is an inappropriate model for assessing the developmental toxicity of flumioxazin in humans because, unlike humans, they are highly sensitive to PPO inhibition, resulting in anaemia in the embryo and consequent developmental toxicity. There was considered to be no plausible 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.

In response to questions from the RAC 28 meeting and ODD, further clarification of the species difference in PPO inhibition and the sensitivity of both rat and human embryonic erythroblasts was provided by the Notifier, as summarised below (refer to Annex 2).

The following conclusions were drawn based on the information presented.

1. The *in vitro* rat REL erythroid cell is an appropriate model for assessing the effects on erythroids *in vivo*

2. Reduced haem biosynthesis is observed in REL cells at a concentration of 0.1 µM, close to that in embryos whose mother was exposed to 30 mg/kg flumioxazin and where PPIX accumulation is observed.

3. In contrast, in human fetal cells (CD36+ derived from cord blood) or human K562 cells, haem biosynthesis is not reduced at concentrations up to 5 µM even though PPIX accumulates at concentrations of 1 µM and above in a dose-dependent manner. The *in vitro* human cells are considered appropriate models for predicting the effects on erythroids *in vivo* in the human adult and fetus.

4. PPO activity could be close to that of the rate-limiting enzyme and reduced PPO activity becomes rate-limiting in rats, but not in humans. The overall difference in inhibitory activity on haem biosynthesis between rats and humans is greater than 50 times (0.1 µM vs 5µM). An expert statement from an independent clinician, Professor Meissner, whose laboratory

has evaluated up to 3000 cases of VP patients over 35 years, noted that there are no reports of the fetus presenting with symptoms of anaemia, nor cardiac malformation, or reports of anaemia in adult VP patients showing a loss of up to 50% of PPO activity (Meissner, 2014 – see Section 4.11.4.3.2).

5. The PBPK model is shown to be a justifiable approach to predict flumioxazin concentrations in the fetus of the pregnant human. The model shows that concentrations of up to 5 µM in human erythroblasts, which do not inhibit haem synthesis or cell proliferation, would far exceed those attained in human embryos following flumioxazin exposure at a maternal dose of 1000 mg/kg.

In conclusion, it is highly unlikely that humans are susceptible to the anaemia in embryos and developmental effects of flumioxazin as seen in rats owing to the substantial qualitative and quantitative species differences between the rat and human.

Subsequently, at the RAC 29 meeting it was concluded that relevance for humans could not be excluded, although there may be quantitative differences between rats and humans. The RAC did not agree that non-relevance was sufficiently shown. On the other hand it was recognised that the CLP Regulation allowed the use of category 2 if there were doubts about the relevance of the MoA to humans. However, the RAC members concluded that the doubts in this case were not sufficient to warrant classification as Repr. 2. In response to this concern the Notifier conducted additional studies to support re-classification (see summaries in Section 4.11.4.3).

4.11.4.3 New mechanistic studies for detailed evaluation by the RAC

New mechanistic studies conducted to address the questions and concerns from the RAC (RAC Opinion proposing harmonised classification and labelling at EU level of Flumioxazin, 06 June 2014). These studies are summarised in detail for evaluation by the RAC below.

4.11.4.3.1 *In vitro*

Table 18: Overview of new *in vitro* mechanistic studies conducted on flumioxazin

Method	Results	Remarks[1]	Reference
Inhibitory action against the enzyme PPO obtained from human liver mitochondria, *in vitro* Flumioxazin: 100 pM - 1 µM 3-OH flumixoazin: 100 pM – 10 µM 4-OH flumioxazin: 1 nM – 100 µM APF: 10 nM - 100 µM No guideline available; non-GLP	**Flumioxazin has the strongest inhibitory activity against PPO among the 4 substances tested, followed by 3-OH flumioxazin and 4-OH flumioxazin, which were 5 and 43 times weaker than flumioxazin. APF does not have any inhibitory activity against PPO up to 100 µM.** These results were broadly comparable with those in rat liver mitochondria (SBT-0118)	2 (reliable with restrictions) **key study** **Flumioxazin purity: 99.4%** **3OH flumioxazin purity: 99.6%** **4OH flumioxazin purity: 99.0%** **APF purity: 99.9%**	Abe, J. (2014) SBT-0128

Method	Results	Remarks[1]	Reference
Comparative effects of flumioxazin and dihydroartemisinin (DHA) on the haem synthetic pathway and cell proliferation in rat erythroleukemia (REL) cells, *in vitro* Flumioxazin: 5.0 µM DHA: 0.125, 0.5, 2.0 µM No guideline available; non-GLP	**PPIX accumulation (↑ up to 30-fold) and reduction of haem content (↓~2-fold) per cell were observed with flumioxazin, however there was no effect on cell proliferation in REL cells.** **DHA caused no accumulation of PPIX in REL cells but caused a reduction of haem content (↓2-3-fold) and inhibition of cell proliferation (↓up to 20-fold) at concentrations of 0.5 and 2.0 µM.**	2 (reliable with restrictions) **Flumioxazin and DHA** **key study** **purity: 99.4%**	Kawamura, S. (2015a) SBT-0132
Comparative effects of flumioxazin and DHA on the haem synthetic pathway and cell proliferation in human K562 cells, *in vitro* Flumioxazin: 5.0 µM DHA: 0.125, 0.5, 2.0 µM No guideline available; non-GLP	**PPIX accumulation in K562 cells (↑ up to 15-fold) was observed with flumioxazin, however there was no effect on cell proliferation or haem content per cell.** **DHA caused no accumulation of PPIX in K562 cells but caused a reduction of haem content (↓up to 4-fold) and inhibition of cell proliferation (↓up to 3-fold) at the highest concentration of 2.0 µM.**	2 (reliable with restrictions) **Flumioxazin and DHA** **key study** **purity: 99.4%**	Kawamura, S. (2015b) SBT-0131
Comparative effects of flumioxazin and DHA on the haem synthetic pathway and cell proliferation in human CD36+ cells, *in vitro* Flumioxazin: 5.0 µM DHA: 0.125, 0.5, 2.0 µM No guideline available; non-GLP	**PPIX accumulation in human CD36+ cells (↑ up to 30-fold) was observed with flumioxazin, however there was no effect on cell proliferation or haem content per cell.** **DHA caused no accumulation of PPIX in human CD36+ cells but caused a reduction of haem content (↓up to 20-fold) and inhibition of cell proliferation (↓up to 25-fold) at ≥0.125 µM and ≥0.5 µM, respectively.**	2 (reliable with restrictions) **Flumioxazin and DHA** **key study** **purity: 99.4%**	Kawamura, S. (2015c) SBT-0130

DHA: dihydroartemisinin
REL: rat erythroleukemia cell line

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

To investigate the inhibitory activity of flumioxazin ([7-fluoro-6-(3,4,5,6-tetrahydro)phthalimido]-4-(2-propynyl)-1,4-benzoxazin-3(2*H*)-one) and its major metabolites, 3-OH flumioxazin, 4-OH flumioxazin and APF against protoporphyrinogen oxidase (PPO), an enzyme inhibition assay was conducted *in vitro* using human liver mitochondrial fraction. The test substances were the same as those used in the comparable study with rat liver mitochondria (SBT-0118) from which purity values were obtained (see Table 15), although no re-analysis of purity was conducted.

Protoporphyrinogen IX was prepared as previously described in the study with rat liver mitochondria (SBT-0118) and is not detailed here. Inhibitory activity of the test substances against the protoporphyrinogen oxidase using protoporphyrinogen IX substrate was conducted according to the previously described method. Concentrations of test substances were as follows:

Flumioxazin:	1 µM, 100 nM, 10 nM, 1 nM, 100 pM
3-OH Flumioxazin:	10 µM, 1 µM, 100 nM, 10 nM, 1 nM, 100 pM

4-OH Flumioxazin: 100 µM, 10 µM, 1 µM, 100 nM, 10 nM, 1 nM
APF: 100 µM, 10 µM, 1 µM, 100 nM, 10 nM

PPO activity of the samples and inhibitory activity of the test substances were evaluated as follows. From the obtained fluorescence-time curves, 30 and 40 minutes were selected as the time points to evaluate the PPO activity, since the plots during 30 to 40 minutes were almost linear in all samples. Fluorescence intensity at the time point was converted to the concentration of protoporphyrin IX (PPIX) with the regression line of the standard samples. Converted PPIX in the heat-killed mitochondria samples was considered to reflect the auto-oxidation of protoporphyrinogen IX, and the amount was subtracted from all samples as BG of the assay system. PPO activity was calculated with the following equations;

Converted amount of substrate during 30 to 40 minutes [pmol/well]
= (PPIX concentration at 40 minutes [µM] – PPIX concentration at 30 minutes [µM])
x Incubation volume (0.1 mL/well) x 1,000

Amount of mitochondria in each sample [mg protein/well]
= Final concentration of mitochondria (0.5 mg protein/mL) x Incubation volume (0.1 mL/well)

PPO activity [pmol/min/mg protein]
= (Converted amount of substrate in the sample [pmol/well]
– Converted amount of substrate in the heat-killed mitochondria samples [pmol/well])
/ Reaction time (10 minutes) / Amount of mitochondria in each sample [mg protein/well]

Vehicle control samples were regarded as full activity control, and the relative PPO activity of each sample was calculated with the following equation;

Relative PPO activity [% of control]
= PPO activity of the sample [pmol/min/mg protein]
/ Mean PPO activity of the vehicle control samples [pmol/min/mg protein] x 100

The concentration-response curves were plotted as relative PPO activity (Y, %) versus logarithm of concentration of the test substances (X, log M). The inhibition curves were fitted to the 4-parameter sigmoidal inhibition model using GraphPad Prism Ver.5 (GraphPad Software Inc.) with weights equal to 1/Y, and the IC_{50} values were obtained. It should be noted that the obtained IC_{50} values were different from EC_{50} values; the concentration of the midpoints of the top plateau and the bottom plateau were obtained from the sigmoidal curves. In addition the concentrations corresponding to 50 % of relative PPO activity vs control were calculated.

The results of the inhibitory activity of the test substances are shown in Table 19 and the calculated IC_{50} values are given in Table 20.

Table 16: Mean relative PPO activity (expressed as % of control) for flumioxazin, 3-OH flumioxazin, 4-OH flumioxazin and APF

Test substance	Concentration [log M]						
	-4	-5	-6	-7	-8	-9	-10
Flumioxazin	-	-	-3.1	18.2	64.6	91.2	93.7
3-OH flumioxazin	-	-5.5	10.3	52.6	77.8	87.1	94.2
4-OH flumioxazin	-7.1	10.9	49.6	73.1	82.9	93.0	-

APF	57.2	68.6	74.4	76.5	84.5	-	-

Table 17: IC_{50} values of flumioxazin, 3-OH flumioxazin, 4-OH flumioxazin and APF against PPO

Test substance		IC_{50} value			
		1st run	2nd run	3rd run	Mean
Flumioxazin	µM	0.024	0.022	0.017	0.021
	[log M]	[-7.62]	[-7.66]	[-7.78]	[-7.68]
3-OH flumioxazin	µM	0.126	0.097	0.089	0.104
	[log M]	[-6.90]	[-7.01]	[-7.05]	[-6.98]
4-OH flumioxazin	µM	0.883	0.495	1.300	0.893
	[log M]	[-6.05]	[-6.31]	[-5.89]	[-6.05]
APF	µM	ND	ND	ND	ND

ND: not determined

The mean IC_{50} value of flumioxazin was 0.021 µM, *i.e.* $10^{-7.68}$ M, 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.* $10^{-6.98}$ M and $10^{-6.05}$ M, and were 5 times and 43 times higher than that of flumioxazin, respectively. These results suggested that PPO inhibitory activity of 3-OH flumioxazin and 4-OH flumioxazin was weaker than that of flumioxazin. An IC_{50} value for APF was not obtained in this study and was determined to be > 100 µM against human PPO.

In conclusion, flumioxazin has the strongest inhibitory activity among the 4 substances tested, followed by 3-OH flumioxazin and 4-OH flumioxazin, which were 5 and 43 times weaker than flumioxazin. APF does not have inhibitory activity against PPO up to 100 µM. The relative potencies of flumioxazin and 3 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.

Comparative effects of flumioxazin and dihydroartemisinin on the haem synthetic pathway and cell proliferation in rat erythroleukemia cells (SBT-0132)

Both flumioxazin and dihydroartemisinin (DHA) cause fetal anaemia which leads to a similar pattern of developmental toxicity in the rat characterized by ventricular septal defects and embryo-fetal death (SBT-00-0012, and Qigui and Weina 2010, respectively). Fetal 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 fetal anaemia by DHA are reported (Finaurini et al., 2012 and Wartenburg et al., 2003).

The rat erythroleukemia (REL) cell line was derived from transplantable tumors from 7,12 dimethylbenz (a) anthracene-induced erythroleukemia in the Long-Evans rat (Kluge et al., 1976). The REL cell line can differentiate into erythrocytes by treatment with various inducer chemicals such as hexamethylenebisacetamide (HMBA) (Yamaguchi et al., 1981). To investigate the effect of flumioxazin and DHA on haem synthesis and protoporphyrin IX accumulation in rat erythroid cells, a study was conducted with REL cells, which were induced to differentiate into erythroid cells by treatment with HMBA.

REL cells were cultured in RPMI medium supplemented with 10 % FBS and 1 % P/S at 37 ºC in a humidified chamber with 5 % CO_2 (CO_2 incubator) and were subcultured every other day. DHA was dissolved in DMSO to prepare a stock solution of 100 mM just prior to the preparation of medium. Flumioxazin was dissolved in DMSO to prepare a stock solution of 200 mM, which was placed in a freezer at -80 ºC until the preparation of medium. RPMI medium containing 40 % FBS, 1 % P/S and 1.0 mM HMBA (HMBA medium) was used as base medium. 20 mM DHA or 50 mM flumioxazin solution were diluted from the stock solution with DMSO, and mixed with HMBA medium to prepare 2.0 µM DHA medium and 5.0 µM flumioxazin medium. 2.0 µM DHA medium was diluted by adding HMBA medium containing 0.01 % DMSO to make test medium containing 0.5 µM and 0.125 µM DHA. HMBA medium containing 0.01 % DMSO was used as the control.

REL cells were centrifuged at 1000 rpm for 5 minutes, re-suspended in each DHA medium (2.0 µM, 0.5 µM, 0.125 µM and 0 µM) or 5.0 µM flumioxazin medium, plated onto 60 mm dishes at 5×10^5 cells/ 5 mL/ dish, and were placed in a CO_2 incubator. The seeding day was defined as Day 0. Control cell suspension at Day 0 was analyzed for haem and PPIX content. On Days 2, 4, 6 and 8, the plated cells were centrifuged at 1000 rpm for 5 minutes, washed with PBS(-) and re-suspended to measure the cell numbers. After that, the cells were centrifuged again, and stored at -80 ºC until measurement. On Days 2, 4, and 6, the cells were subcultured at a concentration of 1×10^5cells/ mL in new dishes at 5mL/ dish. In the 2.0 µM DHA group, the number of cells was too low to subculture at a concentration of 1×10^5cells/ mL on Days 4 and 6, so 1 mL of test medium was added after 1 mL of sampling. There were 3 replicates at each concentration, including the control.

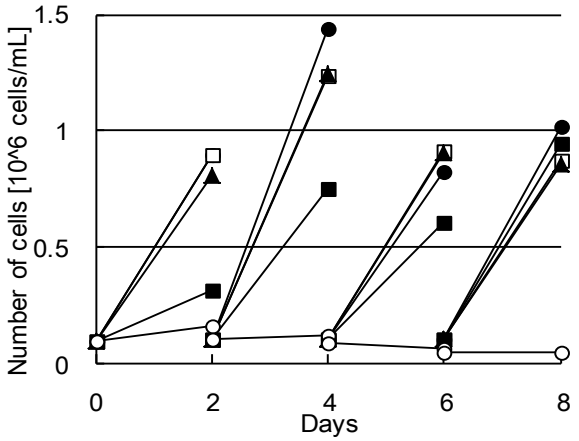
Extraction of PPIX and haem was according to the previously described published method with modifications. After thawing cells at room temperature, 750 µL of basic methanol (methanol/0.1 M ammonia solution = 9/1, v/v) containing internal standards was added to the tubes. As the internal standards for PPIX and haem, 0.2 ng/mL of protoporphyrin IX-d_4 (PPIX-d_4) and 50 ng/mL of deuteroporphyrin IX (DP) were used. Then, the tubes were vortexed for 30 seconds, sonicated for 30 seconds, vortexed again, and centrifuged at 20,000 x g, 4 ºC for 15 minutes. Obtained supernatants were subjected to purification and washing procedures to prepare eluted samples of PPIX, haem and their internal standards for LC/MS analysis according to the previously described published method.

Exposure of differentiated REL cells to flumioxazin at a concentration of 5.0 µM resulted in accumulation of PPIX and the reduction of haem content per cell compared with control but no effect on cell proliferation. These results are consistent with those previously reported (SBT-0125).

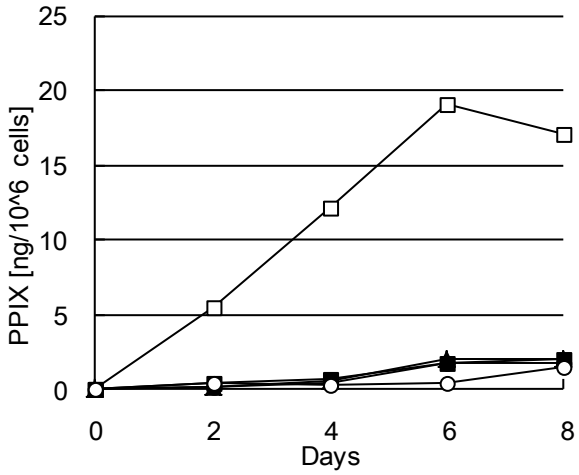
Though 5.0 µM flumioxazin caused the accumulation of PPIX in REL cells from Day 2, DHA caused no PPIX accumulation in REL cells up to 2.0 µM. Dose-dependent reduction of haem content per cell was observed in REL cells at 0.5 µM and 2.0 µM of DHA compared with control. The reduction of haem content reached a maximum at Day 6. Cell proliferation was also inhibited at 0.5 µM of DHA and above in a dose dependent manner from Day 2 The results are summarized graphically in Figure 4.

Figure 4: Comparative effects of flumioxazin and DHA on haem production and proliferation of differentiated REL cells

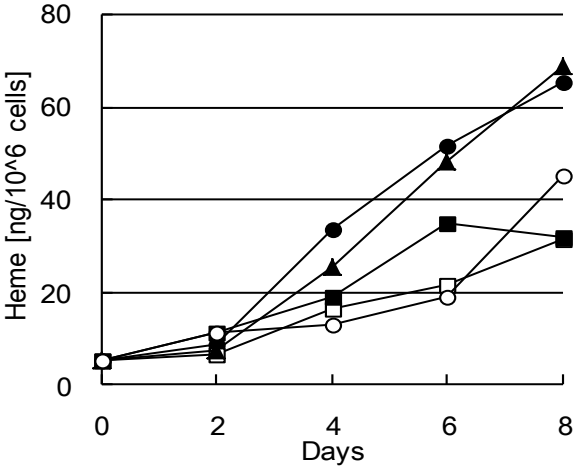
A: Number of cells



B: PPIX concentrations



C: Haem concentrations



5.0 µM of flumioxazin(□), DHA concentrations: 0.125 uM(▲), 0.5 uM(■), 2.0 uM(○), and Control(●)

In summary, 5.0 µM flumioxazin caused the accumulation of PPIX in REL cells although DHA caused no PPIX accumulation in REL cells up to 2.0 µM. The results demonstrate that flumioxazin causes a reduction of haem synthesis resulting from PPO inhibition, but DHA inhibits haem synthesis by a different mechanism. Both flumioxazin and DHA caused developmental toxicity via fetal anaemia in rats and inhibited haem synthesis in the REL cell line. Thus the REL cell line could be a good model to investigate haem synthesis in rats.

Comparative effects of flumioxazin and dihydroartemisinin on the haem synthetic pathway and cell proliferation in human K562 cells (SBT-0131)

Both flumioxazin and dihydroartemisinin (DHA) cause fetal anaemia which leads to a similar pattern of developmental toxicity in the rat characterized by ventricular septal defects and embryo-fetal death (SBT-00-0012, and Qigui and Weina 2010, respectively). Fetal 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 fetal anaemia by DHA are reported (Finaurini et al., 2012 and Wartenburg et al., 2003). In the rat erythroleukemia (REL) cell line, which can differentiate into erythroid cells, flumioxazin and DHA inhibited haem synthesis *in vitro* (SBT-0132). Though flumioxazin causes developmental toxicity specific for rats, DHA causes developmental toxicity not only in rats but also rabbits and monkeys (Qigui and Weina, 2010).

The K562 cell line was derived from a patient with chronic myeloid leukemia in the acute phase (Lozzio, 1975). The K562 cells can differentiate into erythrocytes by treatment with various inducer chemicals such as sodium butyrate (Andersson et al., 1979).To investigate the effect of flumioxazin and DHA on haem synthesis and protoporphyrin IX 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).

K562 cells were cultured in RPMI medium supplemented with 10 % FBS and 1 % P/S at 37 ºC in a humidified chamber with 5 % CO_2 (CO_2 incubator) and were subcultured twice a week. DHA was dissolved in DMSO to prepare a stock solution of 100 mM just prior to the preparation of medium. Flumioxazin was dissolved in DMSO to prepare a stock solution of 200 mM, which was placed in a freezer at -20 ºC before using. RPMI medium containing 1.0 mM NaB (NaB medium) was used as base medium. 20 mM DHA or 50 mM flumioxazin solution were diluted from the stock solution with DMSO, and mixed with NaB medium to prepare 2.0 µM DHA medium and 5.0 µM flumioxazin medium. 2.0 µM DHA medium was diluted by adding NaB medium containing 0.01 % DMSO to make test medium containing 0.5 µM and 0.125 µM DHA. NaB medium containing 0.01 % DMSO was used as the control.

K562 cells were centrifuged at 2000 rpm for 4 minutes, re-suspended in each DHA medium (2.0 µM, 0.5 µM, 0.125 µM and 0 µM) or 5.0 µM flumioxazin medium, plated onto 60 mm dishes at 5×10^5 cells/ 5 mL/ dish, and were placed in a CO_2 incubator. The seeding day was defined as Day 0. Control cell suspension was analyzed for haem and PPIX content. On days 2, 4, 6 and 8, the plated cells were centrifuged at 2000 rpm for 4 minutes, washed with PBS(-) and re-suspended to measure the cell numbers. After that, the cells were centrifuged again, and stored at -80 ºC. On day 4, the cells were subcultured at a concentration of 1×10^5cells/ mL in new dishes at 5mL/ dish. There were 3 replicates at each concentration, including the control.

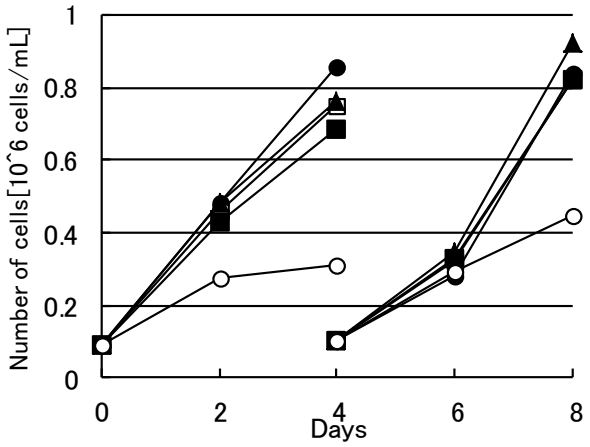
Extraction of PPIX and haem was according to the previously described published method with modifications. After thawing cells at room temperature, 750 µL of basic methanol (methanol/0.1 M ammonia solution = 9/1, v/v) containing internal standards was added to the tubes. As the internal

standards for PPIX and haem, 0.2 ng/mL of protoporphyrin IX-d_4 (PPIX-d_4) and 50 ng/mL of deuteroporphyrin IX (DP) were used. Then, the tubes were vortexed for 30 seconds, sonicated for 30 seconds, vortexed again, and centrifuged at 20,000 x g, 4 ºC for 15 minutes. Obtained supernatants were subjected to purification and washing procedures to prepare eluted samples of PPIX, haem and their internal standards for LC/MS analysis according to the previously described published method.

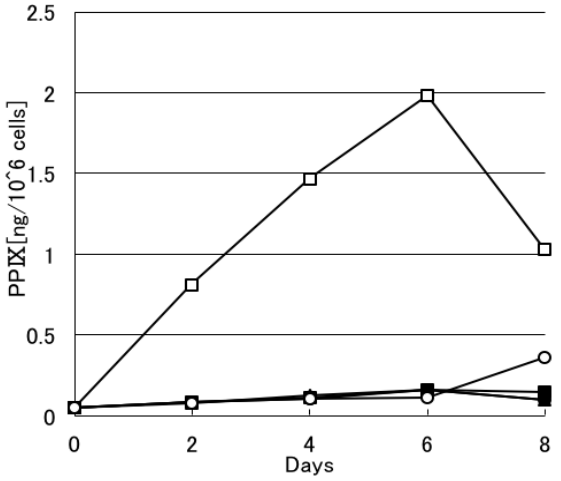
Exposure of differentiated K562 cells to flumioxazin at a concentration of 5.0 μM resulted in accumulation of PPIX but no effect on haem content per cell or cell proliferation. These results were consistent with those previously reported (SBT-0119). DHA caused no accumulation of PPIX in K562 cells. However, reductions of haem content per cell and cell proliferation were observed at 2.0 μM of DHA compared with control. The results are summarized graphically in Figure 5.

Figure 5: Comparative effects of flumioxazin and DHA on haem production and proliferation of differentiated K562 cells

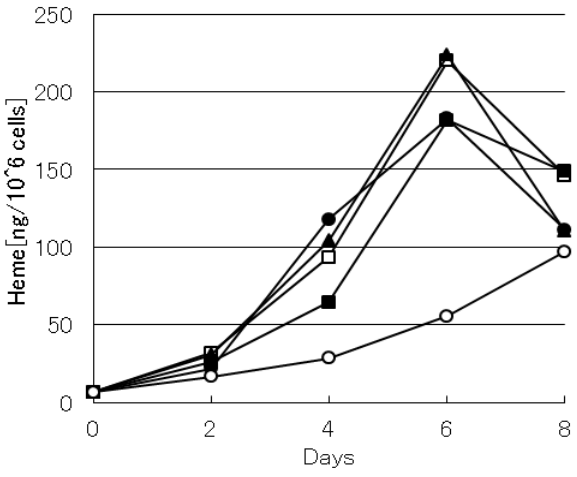
A: Number of cells



B: PPIX concentrations



C: Haem concentrations



5.0 µM of flumioxazin(□), DHA concentrations: 0.125 uM(▲), 0.5 uM(■), 2.0 uM(○), and Control(●)

In summary, DHA caused inhibition of cell proliferation and reduction of haem content per cell in K562 cells consistent with published data (Finaurini et al., 2012). DHA also caused the inhibition of haem synthesis in REL cells (SBT-0132). On the other hand, flumioxazin inhibited haem synthesis in REL cells, but did not inhibit haem synthesis in K562 cells. The results demonstrate that there is 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 on the haem synthetic pathway and cell proliferation in human CD36+ cells (SBT-0130)

In the rat erythroleukemia (REL) cell line, which can differentiate into erythroid cells, flumioxazin and DHA inhibited haem synthesis *in vitro* (SBT-0132). In the human K562 cell line, which can differentiate into erythroid cells, flumioxazin did not inhibit haem synthesis although DHA inhibited haem synthesis *in vitro* (SBT-0131).

In this study, the effect of flumioxazin and DHA on haem synthesis and protoporphyrin IX accumulation was investigated in human CD36+ cells, which are considered to be more physiological than K562 cells (see Section 4.11.5). Human CD36+ cells were derived from human CD34+ cells in culture. Human CD34+ cells were positively isolated from human cord blood using a direct immunomagnetic CD34 MicroBead labeling system and the CD36+ cells were positively isolated using a direct immunomagnetic CD36 MicroBead labeling system. The human CD36+ cells can differentiate into erythroid cells when cultured with stem cell factor (SCF), erythropoietin (EPO), IL-3 and IL-6. This procedure was used to investigate the effect of flumioxazin and dihydroartemisinin (DHA) on the haem synthetic pathway in CD36+ cells differentiated into erythroid cells.

Human CD36+ cells were thawed in Hematopoietic Progenitor Growth Medium (HPGM) supplemented with 25ng/mL SCF, 3U/mL EPO, 10ng/mL IL-3 and 10ng/mL IL-6 and 1 % P/S at 37 ºC and were pre-cultured in a humidified chamber with 5 % CO_2 (CO_2 incubator). DHA was dissolved in DMSO to prepare a stock solution of 100 mM just prior to the preparation of medium. Flumioxazin was dissolved in DMSO to prepare a stock solution of 200 mM, which was placed in a freezer at -80 ºC until the preparation of medium. HPGM containing 25ng/mL SCF, 3U/mL EPO, 10ng/mL IL-3 and 10ng/mL IL-6 and 1 % P/S was used as base medium. 20 mM DHA or 50 mM flumioxazin solution were diluted from the stock solution with DMSO, and mixed with base medium to prepare 2.0 µM DHA medium and 5.0 µM flumioxazin medium. 2.0 µM DHA medium was diluted by adding base medium containing 0.01 % DMSO to make test medium containing 0.5 µM and 0.125 µM DHA. Base medium containing 0.01 % DMSO was used as the control. There were 3 replicates at each concentration, including the control.

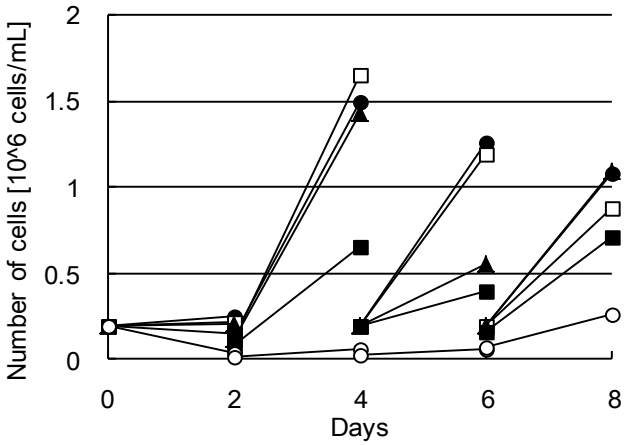
Human CD36+ cells were centrifuged at 2000 rpm for 4 minutes, re-suspended in each DHA medium (2.0 µM, 0.5 µM, 0.125 µM and 0 µM) or 5.0 µM flumioxazin medium, plated onto 30 mm dishes at 6×10^5 cells/ 3 mL/ dish , and were placed in a CO_2 incubator. The seeding day was defined as Day 0. Control cell suspension was analyzed for haem and PPIX content. On days 2, 4, 6 and 8, the plated cells were centrifuged at 2000 rpm for 4 minutes, washed with PBS(-) and re-suspended to measure the cell numbers. After that, the cells were centrifuged again, and stored at -80 ºC. On days 2, 4, and 6, the cells were subcultured at a concentration of 2×10^5cells/ mL in new dishes at 3mL/ dish.

Extraction of PPIX and haem was according to the previously described published method with modifications. After thawing cells at room temperature, 750 μL of basic methanol (methanol/0.1 M ammonia solution = 9/1, v/v) containing internal standards was added to the tubes. As the internal standards for PPIX and haem, 0.2 ng/mL of protoporphyrin IX-d_4 (PPIX-d_4) and 50 ng/mL of deuteroporphyrin IX (DP) were used. Then, the tubes were vortexed for 30 seconds, sonicated for 30 seconds, vortexed again, and centrifuged at 20,000 x g, 4 ºC for 15 minutes. Obtained supernatants were subjected to purification and washing procedures to prepare eluted samples of PPIX, haem and their internal standards for LC/MS analysis according to the previously described published method.

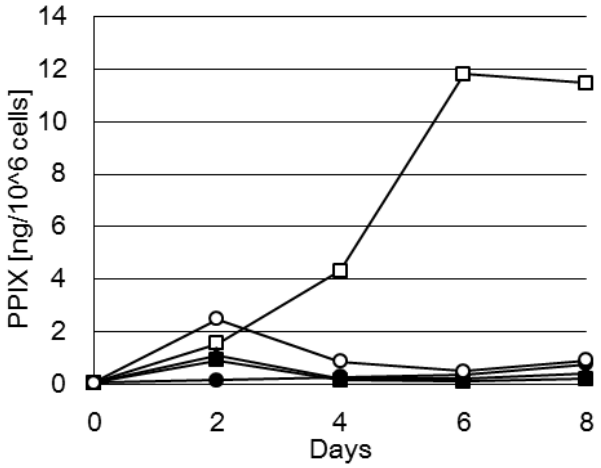
Exposure of differentiated human CD36+ cells to flumioxazin at a concentration of 5.0 μM resulted in accumulation of PPIX but no effect on haem content per cell or cell proliferation. These results were consistent with those previously reported (SBT-0126). DHA caused no accumulation of PPIX in human CD36+ cells. However, a dose-dependent reduction of haem content per cell compared with control was observed at 0.125 μM of DHA and above. Dose dependent inhibition of cell proliferation was observed on all treatment days at 0.5μM and above. On Day 6, 0.125μM of DHA caused the inhibition of cell proliferation. An inhibitory effect at this dose was not observed on Day 2, 4 or 8. The results are summarized graphically in Figure 6.

Figure 6: Comparative effects of flumioxazin and DHA on haem production and proliferation of differentiated CD36+ cells

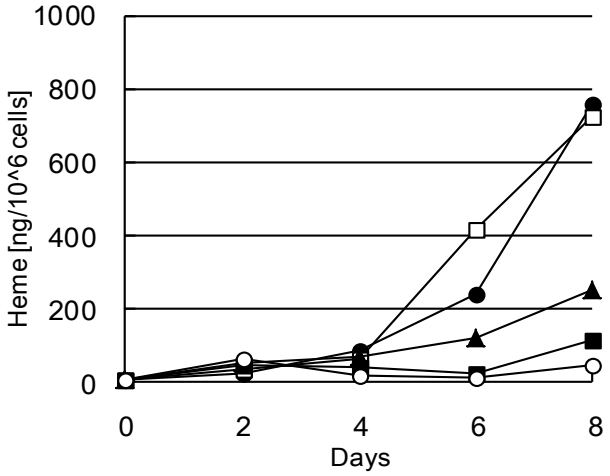
A: Number of cells



B: PPIX concentrations



C: Haem concentrations



5.0 µM of flumioxazin(□), DHA concentrations: 0.125 uM(▲), 0.5 uM(■), 2.0 uM(○), and Control(●)

In summary, DHA caused a reduction in haem content per cell in human CD36+ cells. This result indicates that DHA inhibits haem synthesis in human CD36+ cells. On the other hand, flumioxazin, which caused developmental toxicity specific for rats, inhibited haem synthesis in rat erythroleukemia (REL) cells (SBT-0125), but did not inhibit haem synthesis in human CD36+ cells. These results are consistent with those from the study on human K562 cells (SBT-0131). The results demonstrate that DHA causes the inhibition of haem synthesis but flumioxazin causes no inhibition of haem synthesis in more physiological human cells.

4.11.4.3.2 *In vivo*

Table 18: Overview of new *in vivo* mechanistic studies conducted on flumioxazin

Method	Results	Remarks[1]	Reference
Rat (SD) developmental study investigating embryo-fetal anaemia (20 females/gp) oral: gavage 0, 15, 30, 60 mg/kg/d (exposed GD 6 – 15) No guideline available, non GLP	**Dose dependent anaemia confirmed in gestation day 14 embryos from dams dosed at 30 and 60 mg/kg/d. Anaemia characterised by pale yolk sacs (67/97% at 30/60 mg/kg/d) and pale embryos (51/95% at 30/60 mg/kg/d), and iron deposits in erythroblasts. Very slight anaemia noted at 15 mg/kg/day. Severe anaemia correlated with embryo-fetal lethality (63% post implantation loss on day 20) at 60 mg/kg/d and ventricular septal defects (VSD) at 30 and 60 mg/kg/d (20% and 38% of fetuses).** **30 mg/kg/d established as clear effect dose for anaemia and teratogenicity.** **15 mg/kg/d established as minimal effect dose**	2 (reliable with restrictions) **Flumioxazin** **key study** **purity: 99.6%**	Hosokawa, Y. (2015) SBT-0129

Additional study to evaluate the potential of flumioxazin to cause fetal anaemia at developmentally toxic doses in rats (SBT-0129)

A previous study has shown that flumioxazin induces embryo/fetal lethality and teratogenicity (mainly ventricular septal defects [VSD]) when given orally at a dose of 30 mg/kg/day on gestation days 6-15 (SBT-00-0012). A subsequent mechanistic study showed that a single oral dose of 400 mg/kg on gestation day 12 resulted in severe anaemia in the embryo and fetus and that this is the most likely cause of the observed embryo/fetal 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/day, in order to better understand the cause of flumioxazin-induced embryo/fetal lethality and teratogenicity.

Flumioxazin (purity 99.6%) was suspended in 0.5% (w/v) methylcellulose and administered to three groups of 20 mated (presumed pregnant) female Crl:CD(SD) rats as a single daily oral gavage dose from days 6 through 15 of gestation at dose levels of 15, 30 or 60 mg/kg/day (dose volume 5 mL/kg). The dose levels were chosen based on results of the previous developmental toxicity study (SBT-00-0012). A concurrent control group of 20 pregnant females were similarly dosed with vehicle only. The day on which evidence of mating was seen (presence of vaginal plug *in situ*), was designated day 0 of gestation. The animals were observed daily for mortality and clinical signs of reaction to treatment, and body weights were recorded on gestation days 0, 6, 14 and 20.

On day 14 of gestation, one half of maternal animals were necropsied and their uterine contents were examined. The yolk sacs of all embryos except for the resorbed ones were examined externally to identify and grade anaemia based on the degree of paleness observed. Blood smears were obtained from 2 embryos per litter and stained with Berlin blue to detect abnormal iron deposits in erythroblasts; two hundred erythroblasts from each blood smear were classified into four categories of severity (no positive reaction, a small number of positive granules scattered in the cytoplasm, many fine positive granules in perinuclear cytoplasm, massive deposition of positive granules). Another 2 embryos per litter were examined histopathologically to confirm signs of anaemia or compensatory reactions to anaemia. Four severity grades (slight, mild, moderate and severe) were used to evaluate the histopathological findings. Three severity grades were used to evaluate the erythroblast content in the heart; abundance, depletion and marked depletion. The remaining embryos were examined externally to identify and grade anaemia based on the degree of paleness observed.

The remaining maternal animals were necropsied on day 20 of gestation and their uterine contents were examined. The fetuses were dissected after fixation with Bouin's solution and their hearts were examined to confirm the the reproducibility of developmental effects (VSD) observed in the previous developmental toxicity study.

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/fetal death at 30 and 60 mg/kg/day, but the single instance in the 15 mg/kg/day group was not considered to be treatment-related because there was no post-implantation loss in the dam. Maternal body weight and body weight gain were significantly decreased on day 20 of gestation at 60 mg/kg/day. The effect on maternal body weights was also considered to be due to intrauterine embryo/fetal death. There were no treatment-related effects on maternal body weight or body weight gain at 15 and 30 mg/kg/day. Necropsy of dams on days 14 and 20 of gestation revealed no treatment related macroscopic pathology findings. All females sacrificed on day 14 were pregnant and there were no dams with total litter resorptions. On day 20 of gestation there were 4 dams with total litter resorptions at 60 mg/kg/day and the number of dams with live fetuses was 10, 9, 10 and 6 in the control, 15, 30 and 60 mg/kg/day groups, respectively.

On day 14 of gestation, embryolethality (post-implantation loss) was significantly increased at 60 mg/kg/day, which was attributed to late post-implantation loss. No increase in embryolethality was observed in the 15 or 30 mg/kg/day groups. Paling of yolk sacs and embryos was increased significantly and dose-dependently at 30 and 60 mg/kg/day. Slight increases in pale yolk sacs and pale embryos at 15 mg/kg/day 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/day was very slight, which suggested that flumioxazin caused inhibition of haem synthesis at 30 mg/kg/day and above. The pale yolk sacs and embryos are illustrated in Figure 1. Iron deposits in erythroblasts are illustrated in Figure 2.

Table 19: Selected maternal caesarian section and embryo data (day 14 of gestation)

Parameter	Dose level (mg/kg/day)			
	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 in erythroblasts (%)[1]				
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

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

Figure 1: External observations of yolk sacs and embryos (day 14 of gestation)

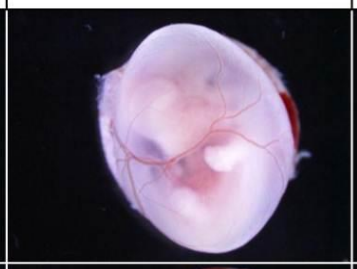
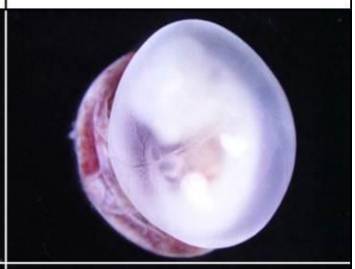
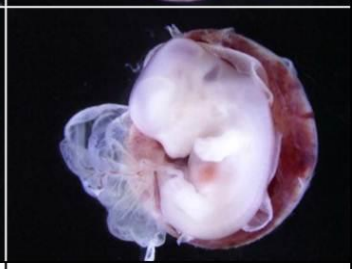
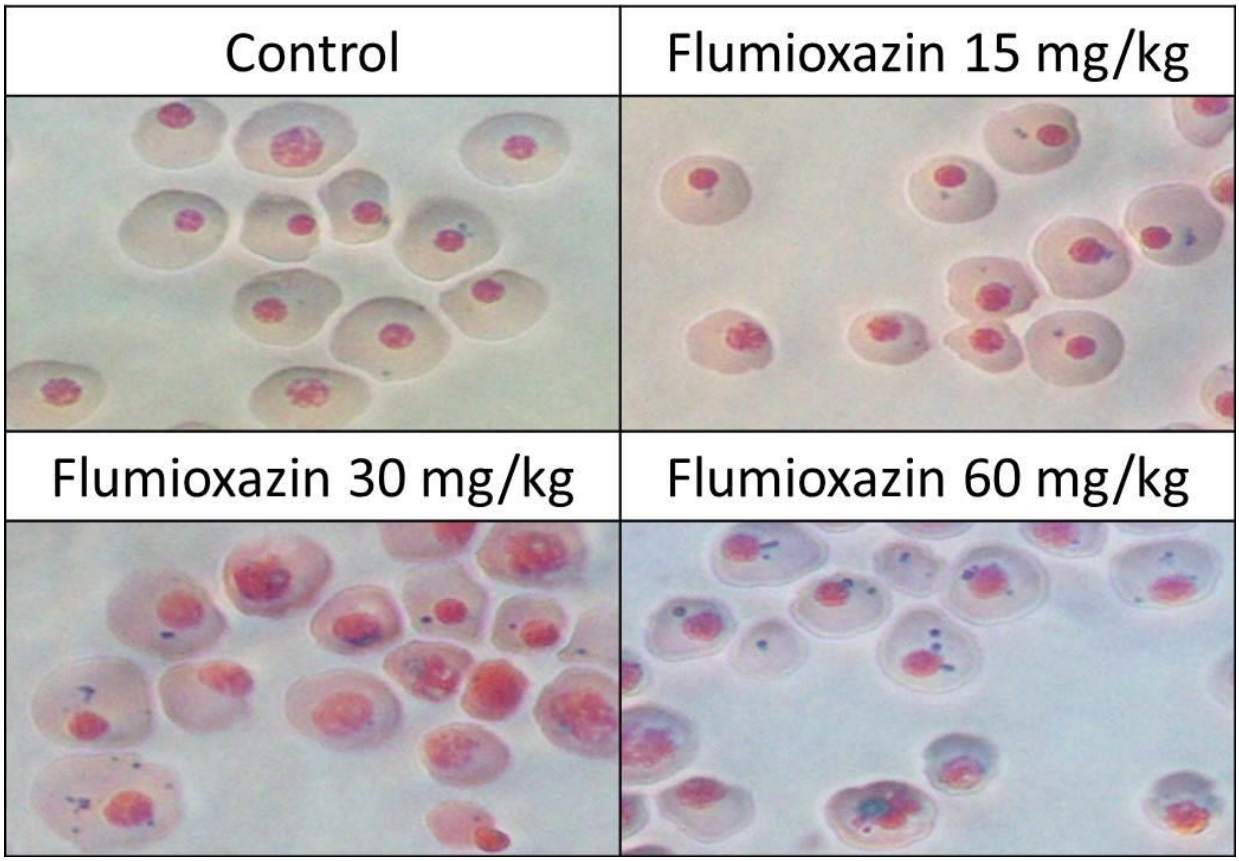
	Normal	Pale	Marked pale
Yolk sac			
Embryo			
Criteria	clear red color of blood vessels, correlating with a abundance of circulating erythroblast	significant reduction in red color in most vessels, correlating with a reduction and/or paling of circulating erythroblast	marked absence of red color in the majority of blood vessels, correlating with a marked reduction of circulating erythroblast

Figure 2: Iron deposits in erythroblasts of embryos (day 14 of gestation)



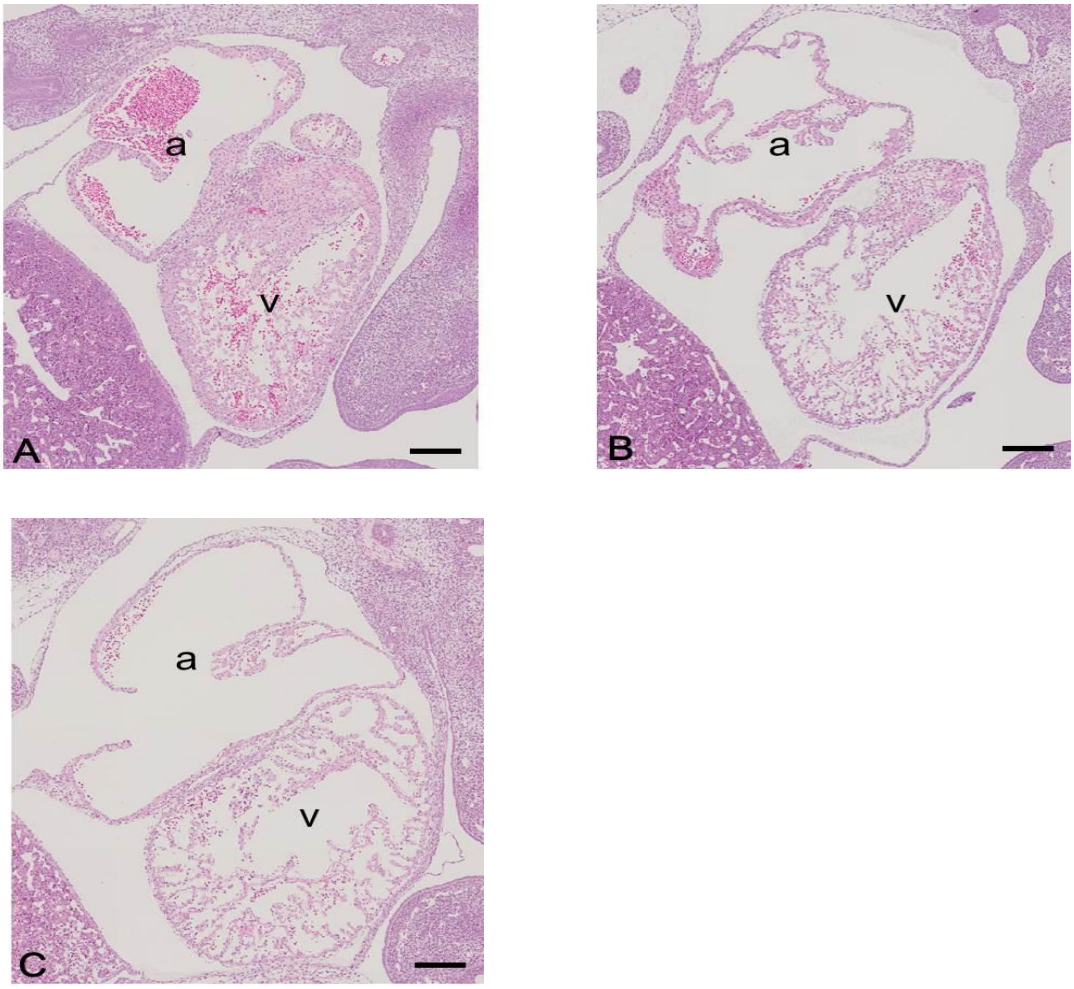
Histopathological changes were observed in the embryonic cardiovascular system. Dose-dependent decreased contents of erythroblasts in the heart and increased degenerative erythroblasts in the liver were observed at 30 and 60 mg/kg/day. These results demonstrated that flumioxazin caused anaemia in the embryos on day 14 of gestation when administered orally to pregnant rats at 30 mg/kg/day and above from day 6 of gestation. In relation to these changes, thinning of the ventricular walls and dilatation of the atrium were observed in the heart of embryos from the 30 and 60 mg/kg/day groups. Moreover, hepatocytic necrosis and dilatation of hepatic sinusoidal vessels were observed at 60 mg/kg/day. Histopathological sections of the heart illustrating the teratogenic findings are shown in Figure 3.

Table 20: Histopathological findings in embryos (day 14 of gestation)

Parameter	Dose level (mg/kg/day)			
	0	15	30	60
Heart - thin ventricular wall (%)				
normal	100.0	100.0	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	100.0	100.0	95.0	80.0 *
slight	0.0	0.0	0.0	0.0
mild	0.0	0.0	5.0	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	100.0	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	100.0	100.0	100.0	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
Hepatocytic necrosis (peripheral regions) (%)				
normal	100.0	100.0	100.0	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

* statistically different from control group $p<0.05$
** statistically different from control group $p<0.01$
Note: there were no gradings of 'severe' for any of the histopathological findings

Figure 3: Histopathological changes in the heart (day 14 of gestation)



A: hematoxylin and eosin-stained sagittal section through a control embryo heart
B: heart from an embryo at 30 mg/kg/day and C: heart from an embryo at 60 mg/kg/day (dilated atrium (a) at 60 mg/kg/day and very thin walls of ventricle (v) at 30 and 60 mg/kg/day)

On day 20 of gestation, embryolethality (post-implantation loss) was significantly increased at 60 mg/kg/day, however, there was no clear treatment related effect at 15 and 30 mg/kg/day. Fetal body weights were significantly decreased in males and females of the 60 mg/kg/day group and those in the 30 mg/kg/day group were also slightly decreased but not significantly.

The number of live fetuses with VSD was significantly increased at 30 and 60 mg/kg/day but not at 15 mg/kg/day. The incidence of VSD at 30 mg/kg/day was 19.7%, which coincides approximately with the result from the previous rat teratology study (25.5% at 30 mg/kg/day) (SBT-00-0012).

Table 21: Selected maternal caesarian section and fetal data (day 20 of gestation)

Parameter	Dose level (mg/kg/day)			
	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 fetuses 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*
Fetal 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 fetuses (%)	20.0	33.3	80.0	83.3*
Number of fetuses with VSD (% of fetuses 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/day and above from days 6 – 15 of gestation and the severe anaemia caused embryo/fetal death and VSD. The clear effect dose for embryonic anaemia and fetal teratogenicity was 30 mg/kg/day and the minimal effect dose was 15 mg/kg/day. The study shows that the same underlying mechanism, anaemia, is likely to be the cause of the adverse developmental effects both at high doses and at the lowest teratogenic dose.

4.11.4.3.3 Human information

Meissner, P. (2014). Flumioxazin and Variegate Porphyria

An expert opinion on human patients presenting with variegate porphyria (VP) in the context of potential adult or fetal 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. This statement was referred to in Annex 2 and was provided to ECHA in February 2014 with the applicant comments on the ODD. It is submitted in **Error! Reference source not found.** of this CLH report and the following summary is provided.

Dr. Meissner is the leading expert in the diagnosis of the porphyrias in South Africa and is a colleague of most porphyria experts around the world. His facility currently diagnoses between 50 and 100 new cases of VP in South Africa. His primary expertise is in the research of the founder gene defect in protoporphyrinogen oxidase (PPO), a mutation commonly referred to as R59W.

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 VP patients show a loss of 50% of activity. About 40% of patients present with clinical symptomology.

By far the majority of patients with VP are able to live a relatively normal lifestyle, and their life-expectancy is normal. There are no reports of pregnancy-related problems in VP mothers related to porphyria. To Dr Meissner's knowledge there are no reports of the fetus presenting with symptoms of anaemia, nor cardiac malformation. Similarly children of VP patients, themselves carrying (or not) the VP 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 VP children nor in adulthood. There are some families in his facility's database whose VP status was monitored from birth to their mid-forties, themselves having produced children. Dr Meissner noted that his comments were not based on formal study but because his facility has been a specialist porphyria centre, and the major referral centre in South Africa, since 1963. This routine follow up has been in place, in some cases for 45 years.

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

4.11.5 Short summary and overall relevance of the provided information on adverse effects on development

An overall summary of the MoA and relevance to humans for the teratogenic effects in the rat is presented below. The following studies were conducted, comprising guideline developmental toxicity studies and mechanistic studies to establish the teratogenic MoA and its relevance to humans.

Table 22: List of developmental and mechanistic studies

Objective	*In vitro* or *in (ex) vivo*	Study type	Reference
Guideline developmental toxicity studies			
To characterise the developmental toxicity of flumioxazin	*In vivo*	Rat (SD) oral developmental study	Kawamura (1990a) SBT-00-0012
		Rabbit (NZW) oral developmental study	Hoberman (1991) SBT-11-0017
		Rat (SD) dermal developmental study	Kawamura (1991) SBT-10-0021
Mechanistic studies to establish the teratogenic MoA in the rat			
To investigate the pharmacokinetics and	*In vivo*	Rat (SD) oral pharmacokinetic study	Takaku (2012a) SBM-0092

Objective	*In vitro* or *in (ex) vivo*	Study type	Reference
placental transfer of flumioxazin		Rat / rabbit (HW / NZW) oral pharmacokinetic study	Shirai (2009) SBM-0081
		Rat / rabbit (SD / JW) study examining placental transfer of flumioxazin	Isobe (1993) SBM-30-0032
		Rat / mouse (SD / ICR) study examining placental transfer of flumioxazin	Isobe (1992) SBM-20-0015
To investigate the critical window and histopathogenesis of critical effects	*In vivo*	Rat (SD) study examining the critical period for developmental toxicity	Kawamura (1993a) SBT-30-0044
		Rat / rabbit (SD / JW) study examining the histopathological effects of flumioxazin on embryonic development	Kawamura & Yoshioka (1997) SBT-0064 and Kawamura (1993b) SBT-30-0043
		Rat (SD) study examining the pathogenesis of developmental effects produced by flumioxazin	Kawamura (1997) SBT-0065
To investigate the developmental effects of flumioxazin analogues	*In vivo*	Rat (SD) developmental study with S-23121	Kawamura (1990b) PPT-00-0023
		Rabbit (NZW) developmental study with S-23121	Hoberman (1990) PPT-01-0020
		Rat (SD) developmental study with S-23031	Lemen (1991a) SAT-11-0024
		Rabbit (NZW) developmental study with S-23031	Lemen (1991b) SAT-11-0025
To investigate the mechanism of haematotoxicity and embryo-fetal anaemia	*Ex vivo*	Development of rat erythroblasts in rat embryos	Ihara (2011) SBT-0117
	In vivo	Rat (SD) study examining the mechanism of haematotoxicity	Yoshida (1996) SBT-0059
	In vitro	Effects of flumioxazin on haem synthetic pathway and cell proliferation in rat erythroleukemia cells	Kawamura (2013b) SBT-0125
	In vivo	Rat (SD) developmental study investigating embryo-fetal anaemia	Hosokawa (2015) SBT-0129
To investigate PPO inhibition and PPIX accumulation	*In vitro*	Inhibitory action against the enzyme PPO obtained from rat liver mitochondria	Abe (2011a) SBT-0118
		SB herbicides on PPO activity in rat and rabbit liver mitochondria	Noda (1995) SBT-0058

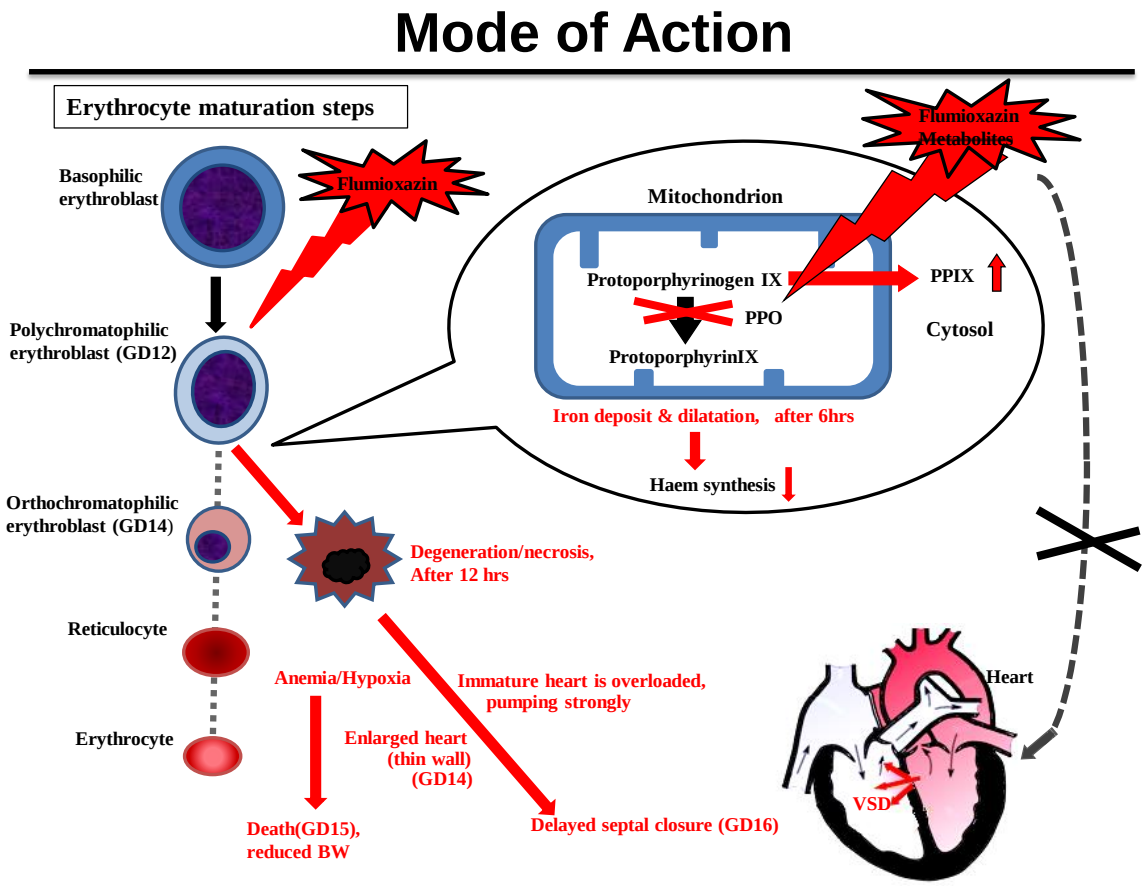
Objective	*In vitro* or *in (ex) vivo*	Study type	Reference
		PPO activity in rat and rabbit tissue	Green & Dabbs (1993) SBT-31-0045
	In vivo	Rat / rabbit (SD / JW) PPIX accumulation in maternal liver and embryos post single administration of flumioxazin	Kawamura (1996a) SBT-0061, and Kawamura (1993c) SBT-30-0042
		Rat (SD) PPIX accumulation in maternal liver and embryos post single administration of flumioxazin and analogues	Kawamura (1996b) SBT-0062, and Kawamura (1993d) SBT-30-0042
		Rat / rabbit (SD / JW) PPIX accumulation in maternal liver and embryos	Kawamura (1996c) SBT-0063, and Kawamura (1993e) SBT-30-0042
Mechanistic studies to investigate relevance to humans			
To investigate species comparison of PPO inhibition and PPIX accumulation	*In vitro*	Species difference in accumulation of PPIX in primary hepatocytes from rat, rabbit, monkey & human	Abe. (2011b) SBT-0120
		Inhibition of PPO by flumioxazin in rat, human and rabbit liver	Green & Dabbs (1996) SBT-0060
		Inhibitory action of flumioxazin and metabolites against the enzyme PPO obtained from human liver mitochondria	Abe (2014) SBT-0128
To investigate effects on human cell lines	*In vitro*	Effects of flumioxazin on haem synthetic pathway and cell proliferation in human CD36+ cells	Kawamura (2013a) SBT-0126
		K562 cell differentiation into erythroid cells in the presence of flumioxazin	Kawamura (2012a) SBT-0119
		K562 cell differentiation into erythroid cells in the presence of metabolites of flumioxazin	Kawamura (2012b) SBT-0123
		Comparative effects of flumioxazin and dihydroartemisinin (DHA) on the haem synthetic pathway and cell proliferation in rat erythroleukemia (REL) cells	Kawamura (2015a) SBT-0132
		Comparative effects of flumioxazin and DHA on the haem synthetic pathway and cell proliferation in human K562 cells	Kawamura (2015b) SBT-0131
		Comparative effects of flumioxazin and DHA on the haem synthetic pathway and cell proliferation in human CD36+ cells	Kawamura (2015c) SBT-0130

Objective	*In vitro* or *in (ex) vivo*	Study type	Reference
To develop a PBPK model for comparative rat and human exposure	*In vitro (and in silico)*	PBPK modelling of flumioxazin in rats and humans	Takaku (2012b) SBM-0093

Flumioxazin induced embryolethality and teratogenicity in the rat following dosing *via* both the oral and dermal routes. Evidence of teratogenicity was exhibited in the form of cardiovascular abnormalities, mainly VSD, and an increase in the incidence of wavy ribs was also observed. Furthermore, fetal growth retardation was also observed in both studies. These effects observed in the rat were in the absence of maternal toxicity. In contrast to the rat, flumioxazin showed no evidence of developmental toxicity in the rabbit even in the presence of maternal toxicity. The maximum dose administered in the rabbit study was 100-fold greater (3000 mg/kg/d) than the maximum dose administered in the rat oral developmental study.

Plausibility of the MoA in the rat

A large body of evidence has been presented to elucidate the MoA for teratogenicity in the rat. This is summarised below:



1. Flumioxazin inhibits PPO which is an enzyme localized in mitochondria. Metabolites do not (SBT-0118).

2. Primitive erythropoiesis starts as a large synchronous wave in the rat (SBT-0117).

3. Polychromatophilic erythroblasts synthesize haem most actively and are the targets of flumioxazin.

4. Critical period is GD 12 (SBT-30-0044). Exposure to flumioxazin at 400 mg/kg on GD 12 induces the highest incidence of VSD, and also other effects (fetal death (GD 15) and growth retardation) (SBT-30-0065). Polychromatophilic erythroblasts are the main erythroid components on GD 12 (SBT-0117) and flumioxazin induces drastic loss of those cells (SBT-0129).

5. Electron microscopy revealed that the first effect site is mitochondria of polychromatophilic erythroblasts. After 6 hrs of exposure to flumioxazin, matrix dilatation and iron deposition were observed in erythroblast mitochondria without any findings in heart tissue including cardiomyocytes and the vascular system (SBT-0064).

6. On GD 12, the heart is undergoing organogenesis. To compensate for anaemia, the heart pumps strongly, and is dilated (thin wall (SBT-0064) or enlarged at necropsy), leading to delay of closure of the interventricular foramen.

7. There is good correlation between strength of PPO inhibition (via PPIX accumulation) and haem synthesis (SBT-0019, 0123) or developmental toxicity (SBT-0062).

8. In the new developmental study, exposure to flumioxazin at 30 and 60 mg/kg/day from GD 6 – 15 induced increased paling of yolk sacs and embryos on GD 14 and VSD in fetuses at necropsy on GD 20, respectively. Blood smears showed iron deposition confirming anaemia (SBT-0129).

9. The former MoA studies were conducted with single high dose administration to clarify the potential toxicity. With the results of the new study (SBT-0129), the proposed MoA has been further elucidated and confirmed to occur at the lowest dose causing VSD.

Possible alternative mechanisms were presented in Appendix 1 of Annex 1 and there was no compelling evidence for a plausible alternative MoA. The new developmental study has demonstrated good dose concordance for embryo-fetal anaemia and teratogenicity at the developmentally toxic dose in the previous developmental toxicity study (SBT-00-0012). In conclusion the proposed MoA in the rat is considered to be plausible and convincing. This has addressed one of the key concerns expressed by the RAC.

Human relevance

The rat embryo is shown to be much more sensitive than the adult regarding the key events leading to anaemia. The RAC was concerned about the potential sensitivity of human embryos to developmental effects even though they may not be the same as those in the rat owing to the apparent species difference in sensitivity to the key events. It was noted earlier that there is a species difference between rat and human in the erythropoiesis pattern during the developmental period (see Section 4.11.4.3). 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. 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 fetal 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 new *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 (Section 4.11.4.3.1.). Flumioxazin caused an accumulation of PPIX in all 3 cell lines. The rat REL cells were the most sensitive and were affected with lower concentrations of flumioxazin compared with the human K562 and CD36+ cells. Flumioxazin also critically only caused inhibition of haem synthesis in the rat REL cells with no effects in human K562 and human CD36+ cells. The inhibition of haem synthesis in REL cells correlates with anaemia induced by flumioxazin in rats. DHA on the other hand, produced clear inhibition of haem synthesis in all 3 cell lines with no accumulation of PPIX. DHA inhibits haem synthesis in human K562 cells by a mechanism unrelated to PPO inhibition. 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 study with human CD36+ cells is directly relevant, since these are physiologically close to primitive human erythroid cells. CD36+ cells are fetal cells derived from cord blood, which is a rich source of haematopoietic progenitor cells. The CD36 antigen is a specific cell surface marker for erythroid cells and CD36+ cells are erythroid progenitor cells (precursors of erythroblasts). In this regard, they 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. After induction, K562 cells accumulate human embryonic haemoglobin, the electrophoretic pattern of K562 cell hemoglobin corresponding closely with that observed in hemoglobins from purified embryonic erythroblasts. In their uninduced state, K562 cells contain fetal and embryonic globin chains, and globin mRNAs. They also lack several surface, enzymatic, and functional properties that are typical of granulocytes, lymphocytes, monocytes, or adult erythroblasts. 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 fetal haematopoietic cells, as opposed to the adult phenotype (Rutherford et al., 1979; Benz et al., 1980). Refer to Annex 4 for a more detailed explanation.

DHA is a human anti-malarial drug which has been used for many years and no reports of VSD or other teratogenic effects have been reported in babies from mothers treated with artemisinins (Mayando et al., 2012; Kovacs et al., 2015). Thus therapeutic doses of DHA are considered to be safe in human pregnancy despite the fact that haem synthesis is inhibited *in vitro* in human K562 cells and CD36+ cells and that developmental toxicity occurs in laboratory animal species, including primates. On this basis 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. This conclusion is supported by the PBPK modelling which demonstrated that the maximum *in vitro* concentration of 5 µM equates approximately to an *in vivo* maternal dose of >1000 mg/kg, a regulatory limit dose.

The RAC was also concerned at the potential effects of accumulation of PPIX, even though it may not lead to anaemia in the human fetus. The expert opinion by Professor Meissner is

important in this regard (Section 4.11.4.3.2 and Annex 3). He noted that in variegate porphyria (VP) patients 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. However, he concluded that in respect of the specific concerns surrounding possible fetal anaemia and cardiac malformation he is unaware of such symptomologies having been reported in VP patients, or their children. Thus it is highly likely that PPO inhibition and/or accumulation of PPIX induced by flumioxazin would not result in developmental effects in humans, especially given that PPO inhibition is not a rate limiting enzyme in humans in contrast to the rat.

In summary, it is concluded that the MoA 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 human K562 cells and CD36+ cells to inhibition of haem synthesis by flumioxazin at a concentration which equates approximately to a maternal *in vivo* dose of >1000 mg/kg, a regulatory limit dose.
- The absence of developmental effects with DHA in humans at therapeutic doses even though haem synthesis is inhibited *in vitro* in human K562 cells and CD36+ cells.
- The absence of developmental effects in human VP patients with significantly decreased PPO activity and that PPO inhibition is not a rate limiting enzyme in humans.

4.11.6 Comparison with the CLP criteria

The implications of the developmental findings in rats exposed to flumioxazin for hazard classification are evaluated using the criteria of Regulation 1272/2008 and the ECHA Guidance on the Application of the CLP Criteria in Regulation (EC) No. 1272/2008 (ECHA, 2011).

Classification of a substance as a reproductive toxicant to humans (Category 1A – known, Category 1B – presumed, or Category 2 – suspected) is based on a weight of evidence approach and expert judgment.

The guidance considers whether maternal toxicity may influence classification but the developmental toxicity associated with flumioxazin was observed in the absence of gross signs of maternal toxicity even though there was likely to be an underlying anaemia in the dams. Given the specificity of the cardiac malformations it is considered unlikely that secondary consequences of maternal anaemia on the developing fetus would have been responsible, rather it is the anaemia in the embryo which is the cause of the embryolethality and VSD. Although fetal growth retardation, reduced ossification and wavy ribs may be associated with overt maternal toxicity this does not seem to be the case with flumioxazin and these effects are likely attributable to the reductions in fetal serum protein.. Therefore, maternal toxicity is considered not to be a confounding factor in determining the classification of flumioxazin.

The guidance also considers whether there is mechanistic information that needs to be evaluated in deciding the classification for reproductive toxicity. The extensive mechanistic research on flumioxazin has clearly demonstrated that:

1. there is convincing evidence for a single MoA causing the developmental toxicity in the rat (confirmed with the new developmental toxicity study), and

2. humans are unlikely to develop anaemia from PPO inhibition and thus would not be susceptible to the MoA causing developmental toxicity in the rat.

The criteria for Categories 1B and 2 are as follows:

Category 1B

"Presumed human reproductive toxicant

The classification of a substance in Category 1B is largely based on data from animal studies. Such data shall provide clear evidence of an adverse effect on sexual function and fertility or on development in the absence of other toxic effects , or if occurring with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of other toxic effects. **However, when there is mechanistic information that raises doubt about the relevance of the effect for humans, classification in Category 2 may be more appropriate**."

Category 2

"Suspected human reproductive toxicant

Substances are classified in Category 2 for reproductive toxicity when there is some evidence from humans or experimental animals, possibly supplemented with other information, of an adverse effect on sexual function and fertility, or on development, and where the evidence is not sufficiently convincing to place the substance in Category 1. If deficiencies in the study make the quality of evidence less convincing, Category 2 could be the more appropriate classification.

Such effects shall have been observed 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 other toxic effects."

It is concluded that the mechanism of developmental toxicity via severe anaemia observed in rats is unlikely to be relevant for human health hazard prediction. A weight of evidence approach is presented below, from which is proposed a revised category for the classification of flumioxazin. The approach follows the principles of an established human relevance framework (HRF) for non-cancer endpoints prepared by the International Programme on Chemical Safety (Boobis et al, 2008). The publication describes a structured weight of evidence approach to assessing the human relevance of a postulated MoA in animals. Whilst the HRF is primarily aimed at chemical risk assessment it is equally applicable to hazard classification where the human relevance of a MoA in animals requires evaluation. The analogous cancer HRF is cited in the ECHA guidance (2011) for the classification of carcinogens (page 299 § 3.6.2.3.).

The non-cancer HRF 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 MoA in animals?

2. Can human relevance of the MoA be reasonably excluded on the basis of fundamental, qualitative differences in key events between experimental animals and humans?

3. Can human relevance of the MoA 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 the answer to question 1 is **yes**. The weight of evidence is sufficient to establish a single MoA for developmental toxicity in the rat, which operates both at a high dose and at the lowest observed teratogenic dose.

The answer to question 2 is **no** for one key event, PPO inhibition. 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-fetal tissues in sensitivity to PPO inhibition. However, the answer to question 2 is **yes** for another key event, 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.

The answer to question 3 is **yes,** based on the evidence for a marked kinetic difference between rats and humans. Although Boobis et al (2008) note that dismissing human relevance based on quantitative differences is likely to be infrequent, they go on to mention 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 demonstrates that there is unlikely to be a plausible scenario whereby human exposure to flumioxazin could cause the developmental toxicity ascribed to the MoA in the rat.

Another publication by Lavelle *et al* (2012) endorses the HRF prepared by the IPCS. Lavelle *et al* present an algorithm for categorising the relevance of animal data for use in human risk assessment (Figure 1 in the paper). It incorporates the principles of the HRF and the relevant aspects of the algorithm are shown below:

Is the MOA established in animals?

- No. Assume relevant to man
- **Yes, see below**

Are the key events plausible in man?

- No. Not relevant to man
- **Yes, for the initial key event of PPO inhibition and "No" for the inhibition of haem synthesis, see below**

Taking into account kinetic and dynamic factors is the animal MOA plausible in man?

- **No. Not relevant to man (in the case of flumioxazin "unlikely to be relevant to man")**
- Yes, directly. Relevant to man
- Yes, with a sensitivity difference. Relevant to man
- Maybe. Assume relevant to man

The elements in bold are applicable to flumioxazin and support the conclusions presented above that, in the case of flumioxazin, the MoA in the rat is unlikely to be relevant to man.

Overall, sufficient evidence is presented **"that raises doubt about the relevance of the effect for humans"**. On this basis it is considered appropriate to re-classify flumioxazin as Category 2 for developmental toxicity.

4.11.7 Adverse effects on or via lactation

Not relevant to this proposal because the developmental effects of flumioxazin in the rat are attributed to *in utero* exposure.

4.11.8 Short summary and overall relevance of the provided information on effects on or via lactation

Not relevant to this proposal.

4.11.9 Comparison with the CLP criteria

Not relevant to this proposal.

4.11.10 Conclusion on classification and labelling for reproductive toxicity

There is a convincing weight of evidence to conclude that flumioxazin is unlikely to present a reproductive hazard to humans and should not be classified for reproductive toxicity Repr. 1B H360D, based on the criteria for classification in Regulation EC 1272/2008. Therefore, re-classification as Repr. 2 H361d is considered to be justified.

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 MoA 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 MoA 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 MoA for the developmental effects and show that the MoA 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 MoA 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 MoA 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 MoA for humans.

Additional key elements

The classification of flumioxazin as Repr. 1B was initially based on three developmental studies summarised in the table.

Table : *Summary table for animals studies on adverse effects on development induced by flumioxazin.*

Method	Results	Reference
EPA OPP 83-3; GLP SD rats 22 females/dose Oral: gavage 0, 1, 3, 10, 30 mg/kg bw/d (from GD 6-15) Reliability: 1	<u>30 mg/kg bw/d</u> No maternal toxicity Reduced number of live foetuses, reduced foetal body weights, increased incidence of cardiac VSD, wavy ribs, curvature of the scapular and reduced ossification of sacrococcygeal vertebral bodies Maternal NOAEL higher than 30 mg/kg bw/d Developmental NOAEL = 10 mg/kg bw/d	Kawamura, 1990a SBT-00-0012

EPA OPP 83-3; GLP NZW rabbits 20 females/dose Oral: gavage 0, 300, 1000, 3000 mg/kg bw/d (from GD 7-19) Reliability: 1	<u>3000 mg/kg bw/d</u> Reductions in maternal body weight gains and relative and absolute food consumption No developmental effects Maternal NOAEL = 1000 mg/kg bw/d Developmental NOAEL higher than 3000 mg/kg bw/d	Hoberman, 1991 SBT-11-0017
EPA OPP 83-3; GLP SD rats 24 females/dose Dermal: occluded (6 h/d) 0, 30, 100, 300 mg/kg/d (from GD 6-15) Reliability: 1	<u>300 mg/kg bw/d</u> No maternal toxicity Reduced number of live foetuses, reduced foetal body weights, increased incidence of cardiac VSD, wavy ribs and reduced ossification of sacrococcygeal vertebral bodies Maternal NOAEL higher than 300 mg/kg bw/d Developmental NOAEL = 100 mg/kg bw/d	Kawamura, 1991 SBT-10-0021

RAC notes that several developmental effects in rats as reduced number of live foetuses, reduced foetal body weights, increased incidence of cardiac VSD, wavy ribs and reduced ossification of sacrococcygeal vertebral bodies were consistently reported in rats after dermal and oral exposure. No developmental toxicity was detected in rabbits at maternally toxic doses up to 100 times higher than those reported as teratogenic for rats.

Additional teratogenicity study (SBT-0136) (NEW STUDY)

During the public consultation a study entitled *"Prenatal Developmental Toxicity Study of Flumioxazin in Rats"* was submitted. This study was not conducted under GLP but used a protocol similar to OECD TG 414 with deviations (no concurrent vehicle controls, two dosed groups instead of at least three, no historical control data to enhance interpretation of study results).

Flumioxazin was administered by oral gavage at 30 and 60 mg/kg bw/d to Crl:CD(SD) rats (20 females per group) from gestation days 6 to 15. No adverse effects were noted in the clinical observation, body weight, adjusted body weight, body weight gain, food consumption, gravid uterine weight, or necropsy in dams in the 30 or 60 mg/kg bw/d groups.

In the 30 mg/kg bw/d group, no adverse effects were noted in the number of corpora lutea or implantations, implantation index, preimplantation loss, number of embryo-foetal deaths, post-implantation loss, number, body weight, or sex ratio of live foetuses. In the 60 mg/kg bw/d group, the number of embryo-foetal deaths and post-implantation loss tended to be high, and the body weight of live foetuses tended to be low.

In the external examination of live foetuses, no anomalies related to the test substance administration were noted. Fixed visceral examination of live foetuses revealed VSD in 5 foetuses (5.93%) in the 30 mg/kg bw/d group and 13 foetuses (12.46%) in the 60 mg/kg bw/d group.

On the basis of these results, oral doses of 30 and 60 mg/kg bw/d of flumioxazin caused no clear maternal toxicity but a dose-dependent increase in the frequency of VSD in the foetuses. RAC notes that this non-GLP study was performed without a control group and other deviations, decreasing the overall interpretation of the study.

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 MoA. 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.

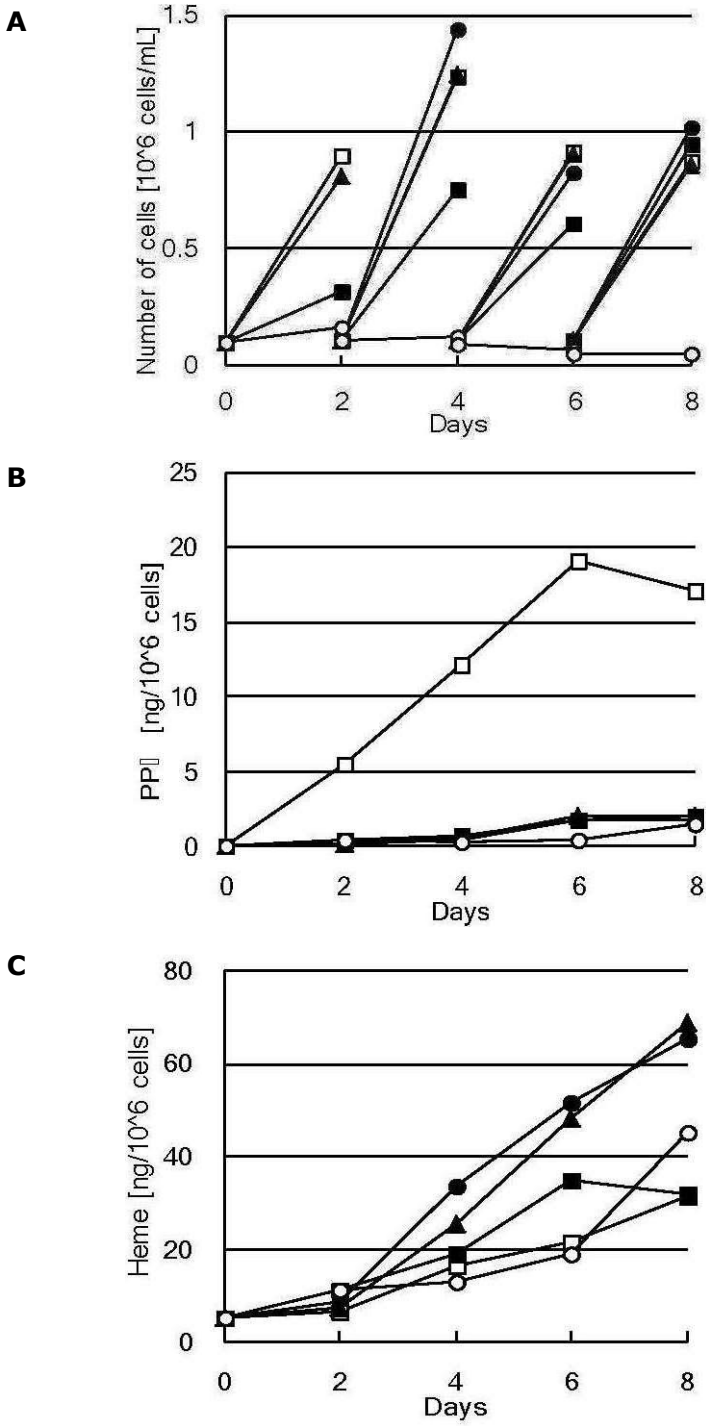


Figure 1: **Comparative effects of flumioxazin and DHA on cell proliferation (A), PPIX accumulation (B) and haem production (C) of differentiated REL cells.** 5.0 µM of flumioxazin (□), DHA concentrations: 0.125 µM (▲), 0.5 µM (■), 2.0 µM (○), and control (●).

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):

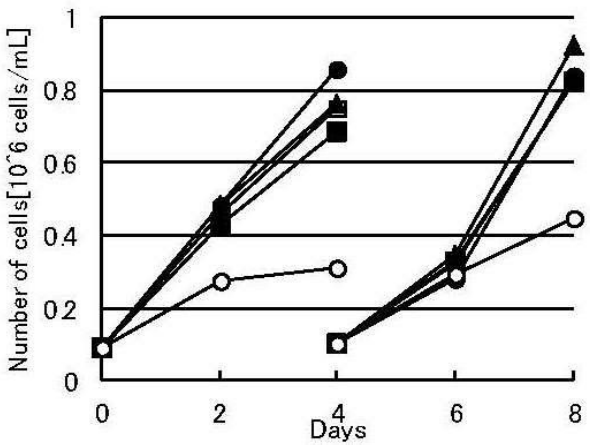
- accumulation of PPIX;
- no effect on haem content per cell compared with controls; and,
- no effect on cell proliferation.

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



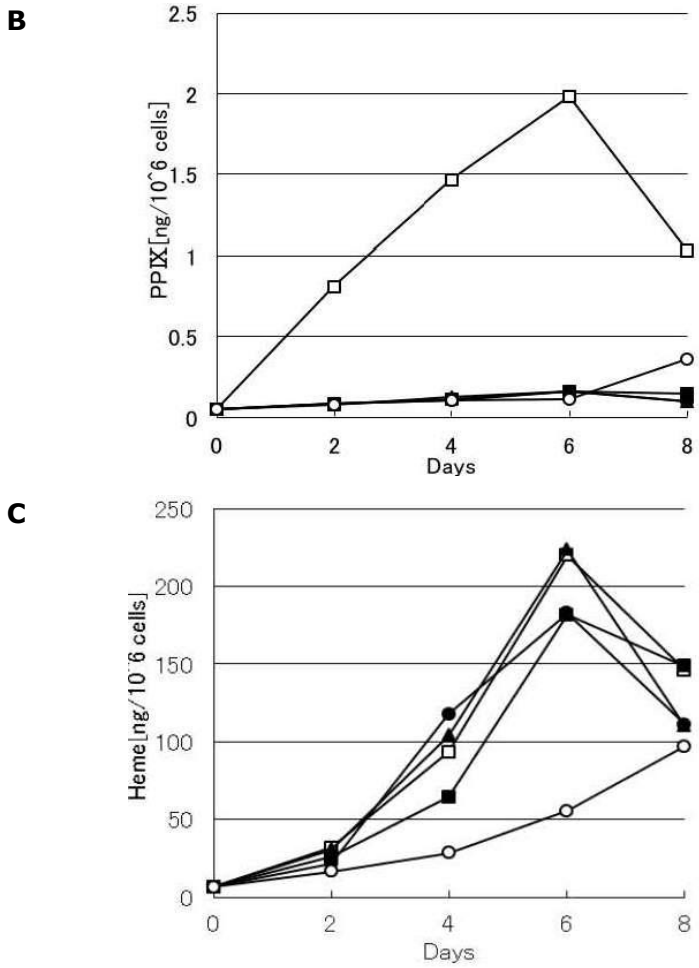


Figure 2: **Comparative effects of flumioxazin and DHA on cell proliferation (A), PPIX accumulation (B) and haem production (C) of differentiated K562 cells.** 5.0 µM of flumioxazin (□), DHA concentrations: 0.125 µM (▲), 0.5 µM (■), 2.0 µM (○), and control (●).

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

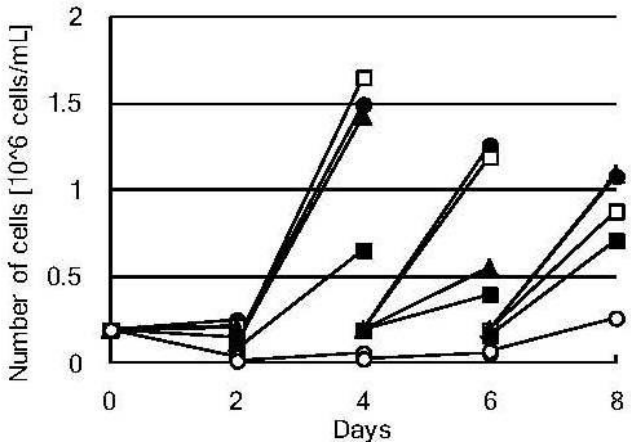
concentration of 5.0 µM resulted in (see also Figure 3):

- 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.

A



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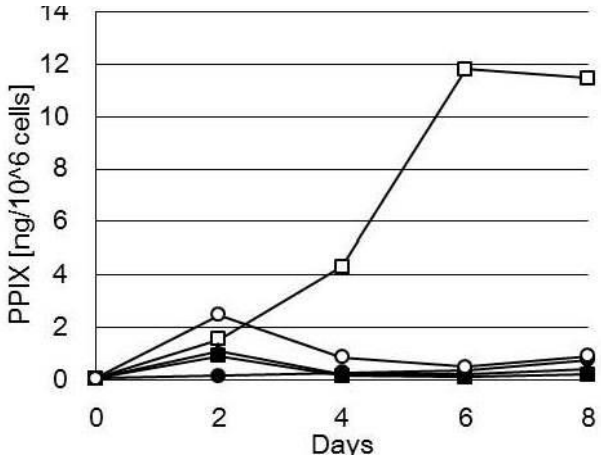

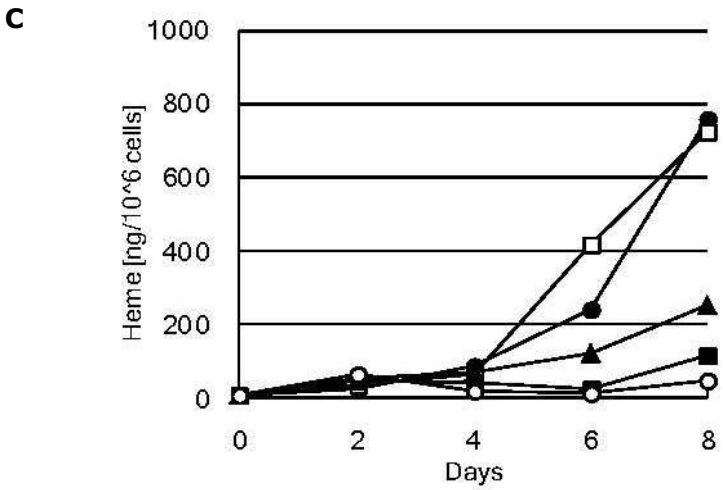

Figure 3: **Comparative effects of flumioxazin and DHA on cell proliferation (A), PPIX accumulation (B) and haem production (C) of differentiated CD36⁺ cells.** 5.0 µM of flumioxazin (□), DHA concentrations: 0.125 µM (▲), 0.5 µM (■), 2.0 µM (○), and control (●).

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).

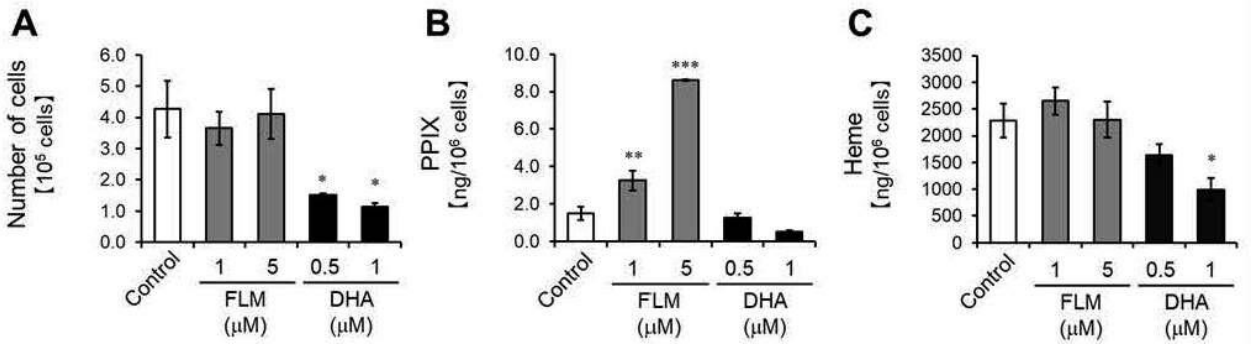

Figure 4: **Evaluation of the effects of flumioxazin and DHA on haem synthesis in erythroids derived from human induced pluripotent cells.** hiPS cell-derived erythroid cells were cultured in differentiation medium from differentiation day 10. Erythroid cells were exposed to flumioxazin, DHA or 0.1% 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).

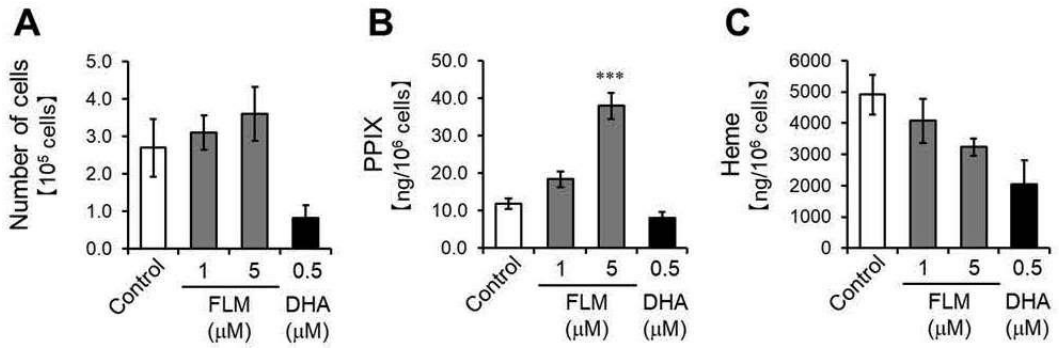

Figure 5: **Evaluation of effects of flumioxazin on haem synthesis in rat embryonic stem cell-derived embryonic erythroid cells.** Rat embryonic stem cells were cultured in differentiation medium for 8 days to produce embryonic stem cell-derived erythroid cells. These erythroid cells were exposed to 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]:				
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.

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 : *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.

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 MoA 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.

Toxicokinetic considerations

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.

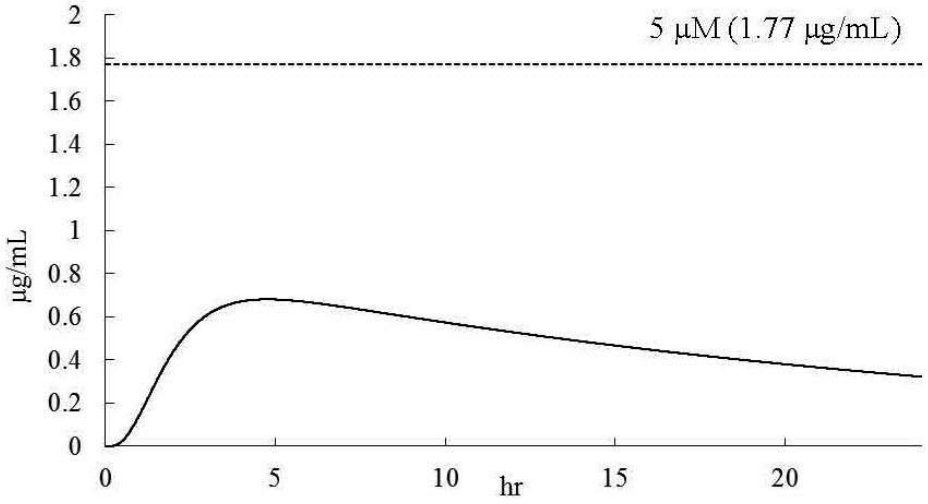

Figure 6: **Calculated flumioxazin concentration in the foetus of pregnant humans orally dosed with flumioxazin at a dose of 1000 mg/kg.**

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 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 MoA 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 MoA 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 7B). 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).

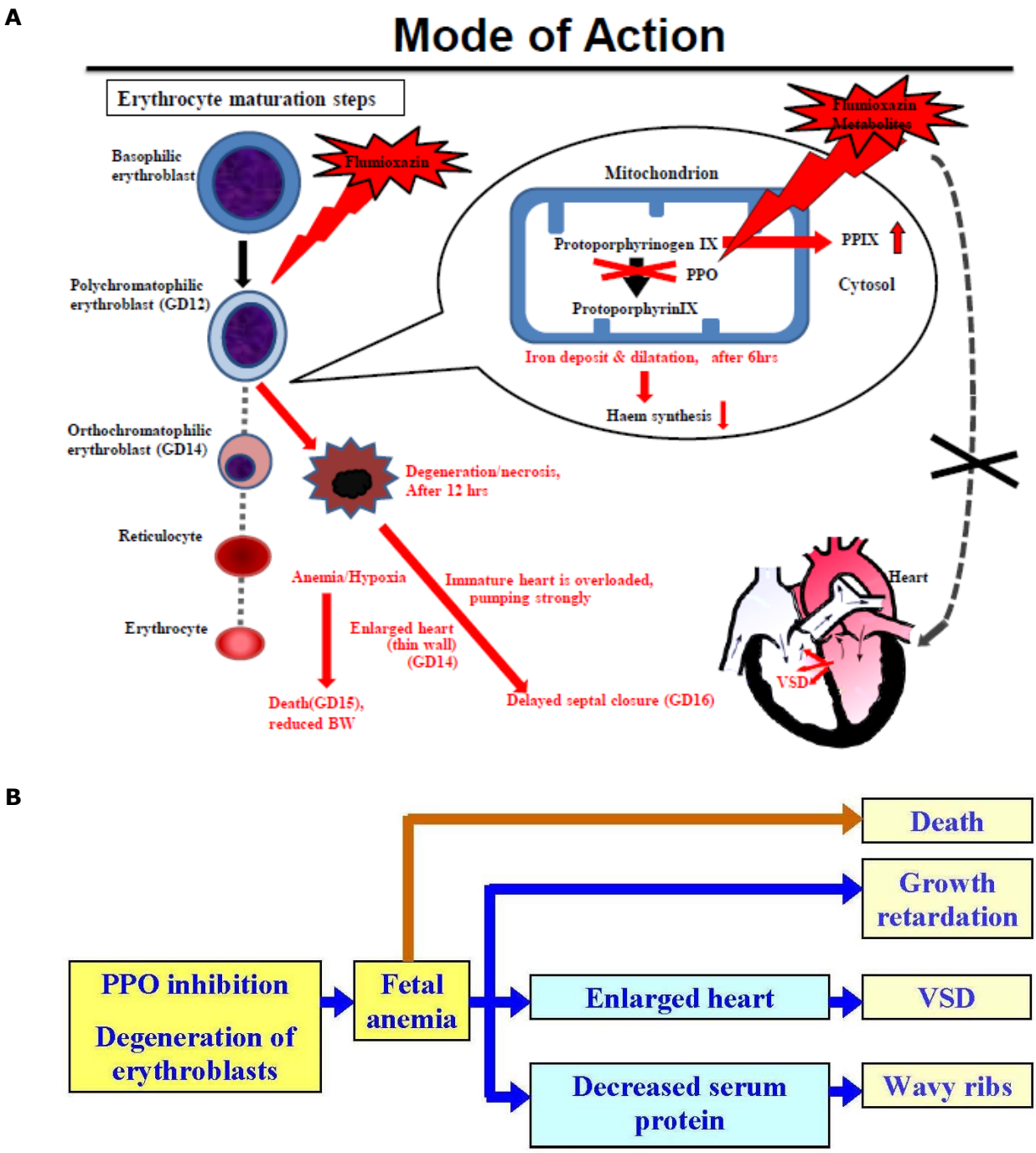

Figure 7: **Mechanism of developmental toxicity induced by flumioxazin.**

Human relevance of the proposed MoA

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 MoA 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 MoA 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 MoA in animals?
2. Can human relevance of the MoA be reasonably excluded on the basis of fundamental, qualitative differences in key events between experimental animals and humans?
3. Can human relevance of the MoA 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 MoA 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 MoA 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)**.

Supplemental information - In depth analyses by RAC

The CLH-dossier contains brief summaries of the studies already reviewed. These studies were summarised in the following tables.

Table: *Previous in vitro Mode of Action studies conducted on flumioxazin.*

Method	Results	Reference
Inhibitory action against the enzyme PPO obtained from rat liver mitochondria, *in vitro* Flumioxazin (99.4%): 10 pM - 1 µM 3-OH flumioxazin (99.4%): 100 pM - 10 µM 4-OH flumioxazin (99.0%): 1 nM - 100 µM APF (99.9%): 1 nM - 100 µM No guideline, no GLP Reliability: 2	Flumioxazin has the strongest inhibitory activity among the 4 substances tested, followed by 3-OH flumioxazin and 4-OH flumioxazin, which were 13.7 and 147 times weaker than flumioxazin. APF does not have any inhibitory activity against PPO up to 100 µM.	Abe, 2011a SBT-0118
Certain herbicides on PPO activity in rat and rabbit liver mitochondria S-53482 (flumioxazin): 94.8% S-23121 (94.7%) S-23031 (94.7%) Reliability: 2	All three SB series herbicides inhibited mammalian PPO activity. The IC_{50} values for S-53482 (flumioxazin), S-23121 & S-23031 were respectively 23, 36 and 2230 nM for rats and 300, 690 and 12500 nM for rabbits. The relative sensitivity of the species to PPO inhibition by SB series herbicides was rat > rabbit.	Noda 1995 SBT-0058
PPO activity in rat and rabbit tissue S-53482 (flumioxazin): 94.8% S-23121 (94.7%) S-23031 (94.7%) Reliability: 2	Adult liver and embryo mitochondria showed similar sensitivity to PPO inhibition by the test compounds, S-53482 (flumioxazin), S-23121 and S-23031, with the rabbit enzyme results showing less sensitivity to inhibition by the test compounds than the rat enzyme. The relative potency for inhibition was flumioxazin (S-53482) > S-23121 > S-23031	Green & Dabbs 1993 SBT-31-0045

Table: *Previous mechanistic in vitro studies conducted on flumioxazin to establish human relevance.*

Method	Results	Reference
Species difference in accumulation of PPIX in primary hepatocytes from rat, rabbit, monkey and human Reliability: 2 Flumioxazin 99.4%	The PPO accumulation as regard the control after treatment with 0.3 µg/ml (≈ 0.8 µM) flumioxazin were 10.3, 1.1, 1.4 and 4.4-fold in primary hepatocytes of rat, rabbit, monkey and human respectively	Abe, 2011b SBT-0120

Inhibition of PPO by flumioxazin in rat, human and rabbit liver Reliability: 2 Flumioxazin 99.4%	The IC_{50} values for flumioxazin after a 20 min incubation period for the inhibition of PPO activity in liver from rats, rabbits and humans were 0.00715 ± 0.0021 µM; 0.138 ± 0.0739 µM and 0.0173 ± 0.0044 µM; respectively.	Green & Dabbs 1996 SBT-0060
K562 cell differentiation into erythroid cells in the presence of flumioxazin Reliability: 2 Flumioxazin purity not stated	PPIX accumulation in K562 cells was observed at concentrations of 1 µM and greater in dose dependent manner, however there was no effect on cell proliferation or haem synthesis at the highest dose tested	Kawamura, 2012a SBT-0119
K562 cell differentiation into erythroid cells in the presence of 5 µM metabolites of flumioxazin Reliability: 2 Purities not stated	There was no effect on PPIX content, haem synthesis or cell proliferation when K562 cells were treated with the metabolites, while flumioxazin increased PPIX in K562 cells	Kawamura, 2012b SBT-0123
PBPK modelling of flumioxazin in rats and humans Reliability: 2 phenyl-U ^{14}C] flumioxazin: 98.6%	The developed human PBPK model demonstrated that the human foetal exposure to flumioxazin following a maternal oral dose of 1000 mg/kg would be 0.68 ppm (1.92 µM), showing that exposure to flumioxazin in a human foetus would be relatively low, even following a maternal dose of 1000 mg/kg.	Takaku, 2012b SBM-0093

Table: *Previous mechanistic in vivo Mode of Action studies conducted on flumioxazin.*

Method	Results	Reference
Rat pharmacokinetic study Crl:CD (SD) rats 3 females/group Oral: gavage Reliability: 2	The total amounts of ^{14}C excreted into bile and urine and ^{14}C remaining in the carcass showed that the absorption (bile + urine + carcass) in females was 12.3% after a single oral administration of flumioxazin at 1000 mg/kg	Takaku, T. (2012a) SBM-0092
Rat /rabbit pharmacokinetic study HW rats NZW rabbits 4 females/group Oral: gavage Reliability: 2	Significantly higher transfer of radioactivity to blood cells in rats compared with rabbits. Elimination of radioactivity from female reproductive tissue of both species was slower than that from plasma, with only a small amount of radioactivity being transferred to the foetus	Shirai, N. 2009 SBM-0081
Rat/rabbit (SD/JW) study examining placental transfer of flumioxazin Oral: gavage	Significantly higher transfer of radioactivity to blood cells was observed in rats compared with rabbits. Elimination of radioactivity from	Isobe (1993) SBM-30-0032

30 mg/kg bw (GD 12 in rats and GD 10 in rabbits) EPA OPP 85-1 Non-GLP Reliability: 2	female reproductive tissue of both species reached maximum 2-4 h after administration and decreased rapidly thereafter, with only a small amount of radioactivity being transferred to the foetus.	
Rat / mouse (SD/ CR) study examining placental transfer of flumioxazin 24 animals/group Oral: gavage 30 mg/kg bw (GD12) EPA OPP 85-1 Non-GLP Reliability: 1	Significantly higher transfer of radioactivity to blood cells was observed in mice compared with rats. Elimination of radioactivity from female reproductive tissue of both species was slower than that from blood (blood cell & plasma), with only a small amount of radioactivity being transferred to the foetus.	Isobe (1992) SBM-20-0015
Rat/rabbit (SD/JW) study examining the histopathological effects of flumioxazin on embryonic development Oral: gavage 0, 1000 mg/kg bw Single exposure on GD 12 EPA OPP 83-3 Non-GLP Reliability: 2	Histopathological changes were restricted to rat embryos only. Microscopy demonstrated mitochondrial lesions including abnormal iron deposition in polychromatophilic erythroblasts and subsequent degeneration of erythroblasts and erythrophagocytosis. Histological changes in the heart, such as thinning ventricular walls followed the erythroblastic lesion. It is concluded that flumioxazin induced VSD by altering haematological function via the inhibition of haem synthesis rather than producing a directly injurious effect on the heart. The embryo compensates for the anaemic hypoxia by increasing heart stroke volume, leading to hypertrophy of the heart. VSD defects result from mechanical distortion of the heart.	Kawamura & Yoshioka, 1997 SBT-0064 and Kawamura, 1993b SBT-30-0043
Rat (SD) study examining the pathogenesis of developmental effects produced by flumioxazin Oral: gavage 0, 400 mg/kg bw	Enlarged heart, oedema and anaemia preceding the occurrence of foetal mortality may be instrumental in the cause of death. Similarly, the occurrence of enlarged heart preceding the failure of the interventricular closure would be related to the pathogenesis of this finding	Kawamura, 1997 SBT-0065

Single exposure on GD 12 EPA OPP 83-3 Non-GLP Reliability: 2		
Rat (SD) study examining the mechanism of haematotoxicity Up to 30 animals/sex/group Oral: feed Study 1 (37 days of exposure): 0, 3000, 10000 ppm Study 2 (15 days of exposure): 0, 3000 ppm	Flumioxazin induced sideroblastic anaemia as result primarily from the defective haem pathway during the process of haemoglobin biosynthesis. The increased blood porphyrin level suggested that flumioxazin induces porphyria	Yoshida, 1996 SBT-0059
Rat/rabbit (SD/JW) PPIX accumulation in maternal liver and embryos post single administration Up to 4 females/group Oral: gavage 1000 mg/kg bw Single dose on GD 12 EPA OPP 83-3 Non-GLP Reliability: 2	PPIX accumulated in rat embryos up to 12 h post dosing, reaching 200-fold greater than the control values. PPIX levels in rabbits remained very low throughout the post dosing period. The species difference in PPIX accumulation in embryos correlates with that of the developmental toxicity produced by flumioxazin.	Kawamura 1996a SBT-0061 and Kawamura 1993c SBT-30-0042
Rat (SD) PPIX accumulation in maternal liver and embryos post single administration Up to 5 females/group Oral: gavage 1000 mg/kg bw Single dose on GD 12 EPA OPP 83-3 Non-GLP	PPIX accumulated in both whole embryos and maternal livers following administration of flumioxazin. The extent of accumulation in embryos was greater than that observed in maternal livers, with the increase of PPIX in the embryos up to 290-fold greater than the control value.	Kawamura 1996b SBT-0062 and Kawamura 1993d SBT-30-0042
Rat/rabbit (SD/JW) PPIX accumulation in maternal liver and embryos Up to 5 females/group Oral: gavage	PPIX accumulated in whole embryos of rats, peaking on GD 11 to 12. Accumulation of PPIX was not observed in maternal rat or rabbit livers or in rabbit embryos.	Kawamura 1996c SBT-0063 and Kawamura 1993e

Rat: 400 mg/kg bw Rabbit: 1000 mg/kg bw Single dose on GD 10-15 EPA OPP 83-3 Non-GLP Reliability: 2		SBT-30-0042

The sub-chronic repeated dose toxicity studies considered relevant to the proposal were provided by the DS as supportive material and were summarised below.

Table: *Summary table for repeated dose toxicity studies in animals with flumioxazin.*

Method	Results	Reference
90 days EPA OPP 82-1 GLP SD rats 12 rats/sex/dose Oral feed 0, 30, 300, 1000, 3000 ppm Males: 0, 2.3, 20.7, 69.7, 243.5 mg/kg bw/d Females: 0, 2.2, 21.7, 71.5, 229.6 mg/kg bw/d Reliability: 1	<u>1000 ppm</u> Mild anaemia and increased relative liver and kidney weights in males. <u>3000 ppm</u> Significant and more marked anaemia with increased incidences of extramedullary haematopoiesis in the spleen and increased absolute spleen weights, relative liver and spleen weights in both males and females Increased absolute thyroid weight and relative heart and thyroid weights in females Increased relative kidney weights in males NOAEL: 300 ppm LOAEL: 1000 ppm	Hagiwara 1989 SBT-91-0002
90 days EPA OPP 82-1 GLP SD rats 10 rats/sex/dose Oral feed 0, 30, 300, 1000, 3000 ppm Males: 0, 1.9, 19.3, 65.0, 196.7 mg/kg bw/d Females: 0, 2.2, 22.4, 72.9, 218.4 mg/kg bw/d	<u>3000 ppm</u> Marked anaemia with increases in spleen, liver, heart and kidney weights in females, and increases in liver, heart, kidney and thyroid weights in males <u>1000 ppm</u> Anaemia and extramedullary haematopoiesis in spleen, increased liver, heart, kidney and thyroid weights <u>300 ppm</u> No effects in males Anaemia and extramedullary haematopoiesis in the Spleen in females NOAEL: 300 ppm (males) and 30 ppm (females)	Adachi 1991 SBT-10-0023

Reliability: 1	LOAEL: 1000 ppm (males) and 300 ppm (females)	
21 days EPA OPP 82-2, GLP SD rat 5 rats/sex/dose Dermal: semi-occluded 0, 100, 300, 1000 mg/kg bw/d Reliability: 1	<u>1000 mg/kg bw/d</u> No adverse effects observed in males and decrease of mean haemoglobin and haematocrit values in females NOAEL: Higher than 1000/300 mg/kg bw/d (male/female) LOAEL: Higher than 1000/1000 mg/kg bw/d (male/female)	Osheroff 1991 SBT-11-0026
52 or 104 weeks EPA OPP 83-5, GLP SD rat 50 rats/sex/group Oral: feed 0, 50, 500, 1000 ppm Males: 0, 1.8, 18.0, 36.5 mg/kg bw/d Females: 0, 2.2, 21.8, 43.6 mg/kg bw/d Reliability: 1	<u>Non-neoplastic effects at 500 ppm and higher doses</u> Chronic nephropathy (male) and haematological changes (anaemia, both genders) NOAEL: 50 ppm LOAEL: 500 ppm	Seki (1993) SBT-30-0040

Moreover, it is also noted that there was no evidence of anaemia in 90-day and 1-year oral toxicity studies in the dog up to the highest dose level of 1000 mg/kg bw/d and in a 28-day oral study in the mouse up to 10000 ppm.

In conclusion, RAC notes that irrespective of route of administration (oral or dermal) the toxic changes observed following flumioxazin exposure in rats were associated to changes in haematopoietic system (mainly anaemia). RAC also notes that rat seems to be the most sensitive species since no anaemia could be detected in dogs and mice at doses up to 1000 mg/kg bw/d.

4.12 Other effects

4.12.1 Non-human information

4.12.1.1 Neurotoxicity

Not relevant to this proposal.

4.12.1.2 Immunotoxicity

Not relevant to this proposal.

4.12.1.3 Specific investigations: other studies

Not relevant to this proposal.

4.12.1.4 Human information

None.

5 ENVIRONMENTAL HAZARD ASSESSMENT

Not relevant to this proposal.

6 OTHER INFORMATION

None.

7 RMS CONCLUSION

RMS keeps the opinion that the mechanism of foetal anaemia - that is a critical precursor of clear developmental toxicity of flumioxazin in rat - is not relevant for humans, and **flumioxazin should be classified as Repr. 2, H361d**. This view is supported by the evaluation of new studies submitted with the new CLH document.

New i*n vivo* study in rats provides the missing evidence of clear anaemia in foetuses of rats dosed repeatedly in GD 6 – 15 with flumioxazin critical daily dose of 30 mg/kg bw but of only minimal effects of a dose of 15 mg/kg bw. The endpoints used (increased deposition of Fe in erythroblasts, decreased number of erythroblasts in the heart, increased number of degraded erythroblasts in liver, changes of colour of yolk sack and embryo) are valid measures of resulting anaemia.

While a direct comparison with human subjects in *in vivo* experiments is not feasible, *in vitro* studies confirmed substantial differences and are supported by three new studies comparing effects of flumioxazin and artemizin on inhibition of haem synthesis and on cumulation of protoporphyrin in two types of human cells (CD36+ cells from human cord blood and K562 human erythroleukemia cells) and rat erythroleukemia cells:

Flumioxazin µM	human CD36+		human K562		rat erythrol.	
	PPIX	Haem	PPIX	Haem	PPIX	Haem
0	100%	100%	100%	100%	100%	100%
0.01	150	96	100	93	95	91
0.1	135	124	95	89	176	72

0.3					308	67
1	239	106	232	108	944	47
5	1186	86	1579	100	2222	37

Unlike flumioxazin, artemisinin inhibited haem synthesis in both human and rat cells, and had no effect on cumulation of PPIX.

Epidemiological or clinical evidence of flumioxazin effects in humans is missing, fortunately. Ample clinical experience with diagnosing and treatment of heterozygotic porphyria variegata patients substitutes at least partly this lack of human data.

Manifest anaemia is not found in porphyria variegata patients, neither in latent state (average activity of protoporphyrinogen oxidase is about 50% of activity in healthy subjects) nor in porphyric crises: lower activity of PPO in porphyria variegata patients is known to be compensated by an increased activity of the synthetase of delta aminolevulinic acid (by feedback form haem level) and possibly by some supplementary mechanisms. On the other hand, lasting increase in the synthesis of delta aminolevulinic acid leads to more rapid accumulation of toxic porphyrin metabolites; intravenous treatment with haem is therapy of choice for porphyria variegata crisis, notwithstanding the fact that no anaemia is found.

Porphyria variegata is genetically extremely homogenous in thousands of affected patients in South Africa. Unlike this situation, cases of VP in other states (e.g. in Sweden or Finland) are caused by tens distinct types of mutations; absence of anaemia also in them supports general inability of PPO inhibition to produce clinically relevant anaemia in human subjects.

Sequencing analysis revealed 10 different mutations in the PPOX gene in 14 out of 17 apparently unrelated Swedish VP families (Wiman et al. 2003). About 80 individuals, representing 28 apparently unrelated families, have been diagnosed with VP in Sweden (Porphyria Centre Sweden), giving a prevalence of approximately 1 : 100 000 inhabitants. One mutation identified in two of Swedish families investigated has also been identified in 11 Finnish VP families, as well as in a French VP family. Prevalence has been estimated to be 1.3 per 100,000 in Finland (Mustajoki 1980) and 0.3 per 100,000 and 0.3 in Europe generally.

Homozygous VP is very rare disease and Poblete-Gutierrez et al.(2006) stated that only 11 homozygous VP patients have been published. Three patients with 5~15% of control PPO activity had normal haemoglobin contents. One patient with 12% of control PPO activity showed dyserythropoietic anaemia by bone marrow examination without changes in peripheral blood picture (Frerrer 2009).

Careful weighing of consequences should take into account that - unlike many other pesticides - flumioxazin residues in human food and animal feed items are all below the limit of quantification, and that - consequently - significant exposure of general population and especially prenatal exposure of human foetuses could be only accidental.

8 ABBREVIATIONS

ATP of the DSD	Adaptation to the Technical Progress of the Dangerous Substances Directive (67/548/EEC)
AV	Atrioventricular
bw	Bodyweight
CLP	Regulation (EC) 1272/2008 of the European Parliament on the Classification Labelling & Packaging of substances and mixtures.
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
ECHA	European Chemicals Agency
EDD	embryonic development day
GD	gestation day
GLP	good laboratory practice
GOT	glutamic-oxaloacetic transaminase
Hb	Haemoglobin
HPLC	High Performance Liquid Chromatography
HRF	human relevance framework
HW	Han Wistar
IPCS	International Programme on Chemical Safety
JW	Japanese white
LC/MS	liquid chromatography/ mass spectrometry
M/E	myeloid / erythroid
MCH	mean corpuscular haemoglobin
MCHC	mean corpuscular haemoglobin concentration
MCV	mean corpuscular volume
mg/kg	milligrams per kilogram
mg/kg/d	milligrams per kilogram per day
MoA	mode of action
NaB	sodium butyrate
NOAEL	no observed adverse effect level
NZW	New Zealand white
PBPK	physiologically based pharmacokinetic
PCE	polychromatophilic erythroblast
ppm	parts per million

PPO	protoporphyrinogen oxidase
PPIX	protoporphyrin IX
RBC	red blood cell
S-53482	Flumioxazin
sd	standard deviation
SD	Sprague Dawley
VSD	ventricular septal defects
WBC	white blood cell

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ANNEXES

ANNEX 1
MECHANISM OF THE DEVELOPMENTAL EFFECTS INDUCED BY FLUMIOXAZIN IN RATS

1. Mechanism of the developmental effects induced by flumioxazin in rats

Substance 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

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Mechanism of the developmental effects induced by flumioxazin in rats

Executive summary

There is convincing evidence for a single mode of action causing the developmental toxicities in rats. The sequence of key biological events in the proposed mode of action has been elucidated at higher dose levels than the developmental toxicity study. It is not uncommon in the teratology research. Observed types and incidences of developmental toxicity by flumioxazin are identical between repeated doses of 30 mg/kg and a single dose of 400 mg/kg. Considering a decrease in absorption rate with increment in oral dose, difference in internal dose levels would not be as large as those calculated from dose levels orally administered. As a result of calculation with the PBPK model, Cmax and AUC of 400 mg/kg are 4 times and 6 times larger than that of 30 mg/kg. A single treatment at 400 mg/kg would be comparable to a repeated treatment at 30 mg/kg during the sensitive period. The evidence supports the identical mode of action to be the basis of developmental toxicities between the developmental toxicity study at 30 mg/kg andthe sensitive period-finding study at 400 mg/kg.

Rat embryos are susceptible to flumioxazin exposure within a shorter period of time than adults due to a single wave of synchronous maturation of rat primitive erythroblasts in yolk sac while PPO inhibition is comparable between adults and embryos. Even, fetuses are less sensitive to flumioxazin once passing the peak sensitive period since a major site of hematopoiesis shifts to the liver and continuing hematopoiesis occurs. In adults, continuing hematopoiesis and remaining erythroblasts non-sensitive to flumioxazin can compensate for effects of flumioxazin on sensitive erythroblasts during a period of compensable time. Thus, the fetus would develop severe anaemia during the sensitive period in the absence of significant anaemia in the adult during several days of exposures to flumioxazin.

Taking all data available into consideration, the mechanism of developmental toxicity by flumioxazin is elucidated and convincing.

Contents

ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON FLUMIOXAZIN (ISO); N-(7-FLUORO-3,4-DIHYDRO-3-OXO-4-PROP-2-YNYL-2H-1,4-BENZOXAZIN-6-YL)CYCLOHEX-1-ENE-1,2-DICARBOXIMIDE

1. Introduction

This document has been prepared to address the points raised in the RAC Opinion Development Document (ODD) and those arising at the RAC meeting (13/03/2014). The additional information presented in this document focuses on the two key areas of uncertainty in the ODD relating to the plausibility of the mechanism of developmental effects induced by flumioxazin in the rat, reproduced below:

B. Mechanism of action

The mechanism is considered plausible on the basis of the following supporting data:

-demonstration of a critical window for all developmental toxicity endpoints

-correlation between inhibition of PPO (via PPIX accumulation) and developmental toxicity via comparision of rat and rabbit and compound-specific differences between flumioxazin and chemical analogues.

-histological evidence of mitrochondrial lesions in polychromatophilic erythroblasts and cardiac damage in rat foetus (compared to no effect in rabbits).

-demonstration of the pathogenesis of the developmental effect in rat (no effect in rabbit).

***However**, some information is incomplete and not always consistent;*

- The critical events were elucidated at significantly higher doses than the dev tox study and were not demonstrated in the rat foetus when the dams are dosed with 30 mg/kg bw (rat dev tox study). Little or no maternal haematoxicity is likely after 6-10 days at this dose considering the 90-day subchronic data.

- In addition, the PPO inhibition data indicate similar IC50 values for adult and foetal rat (ref SBT-0122), yet the foetus is far more sensitive to flumioxazin damage. In contrast, PPIX was demonstrated to accumulate in rat foetus at very high levels compared to adult liver. Placental transfer data and the rat pharmacokinetic study (Shirai 2009) indicate that a very small fraction of the maternal dose reaches the rat foetus. Similar mechanism is likely to occur in humans.

2. Mechanism of action of developmental toxicity by flumioxazin

The mechanism of the developmental toxicity of flumioxazin in rats is presented in Figure 2:

a) Flumioxazin inhibits PPO, which is the penultimate enzyme in haem biosynthesis and is localized in mitochondria (Fig.1). Because of abnormal subcellular location, the resulting PPIX is beyond reach of ferrochelatase and cannot be transformed to haem. Thus inhibition of PPO in the rat embryo results in degeneration of fetal erythroblasts leading to anaemia.

b) Severe fetal anaemia leads to the fetal death.

c) Surviving fetuses are growth-retarded as indicated by a decrease in body weight. 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. This results in delayed closure of the foramen represented as ventral septal defects (VSD) in the term fetus due to mechanical distortion of the heart or abnormal blood flow.

d) Concurrently serum protein is decreased in the fetus resulting in wavy ribs.

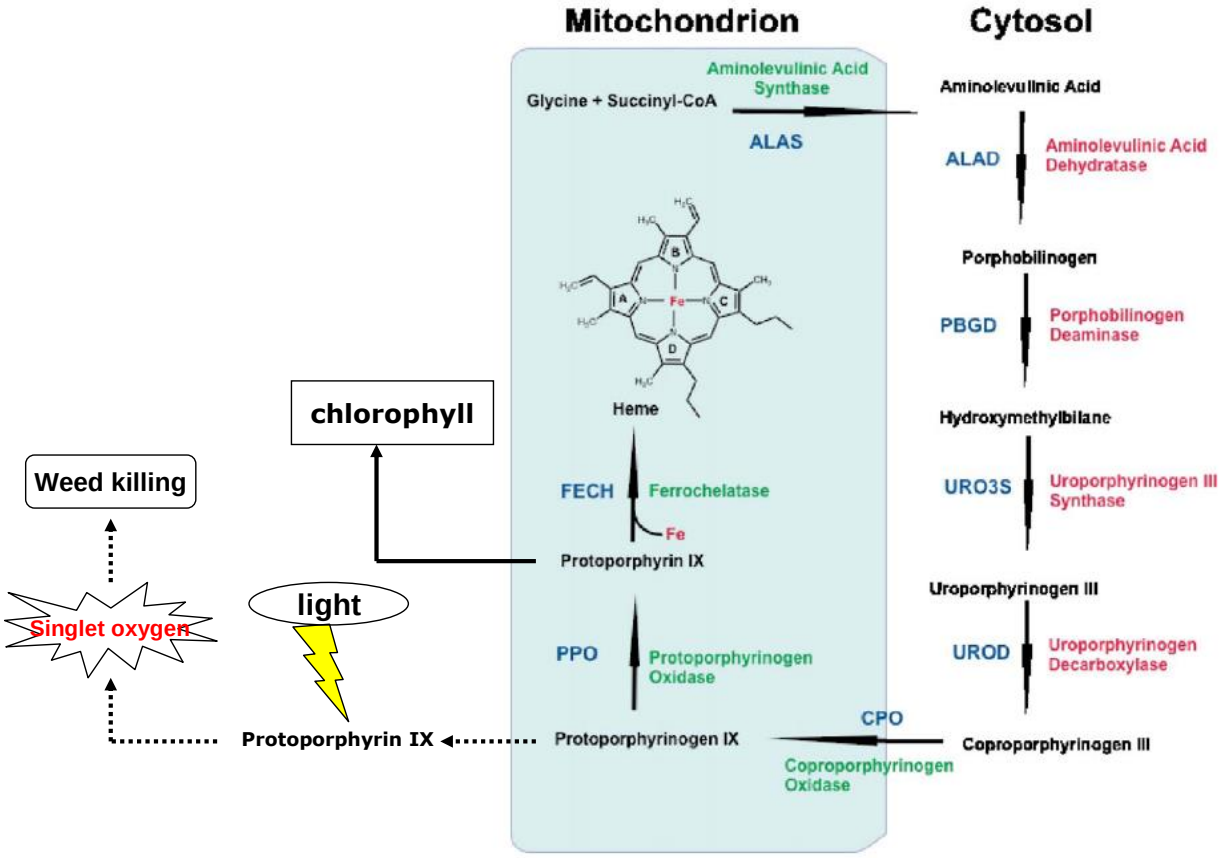

Fig. 1 Heme biosynthetic pathway and mode of herbicidal action of flumioxazin

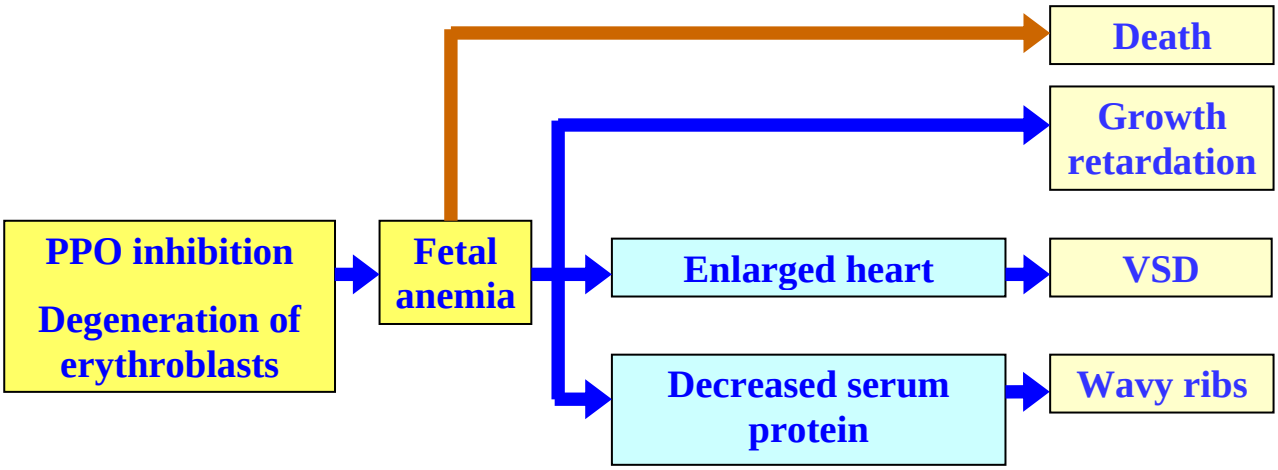

Fig. 2 Mechanism of developmental toxicity induced by flumioxazin

There is convincing evidence for a single mode of action causing the developmental toxicities in rats. The sequence of key biological events in the proposed mode of action has been elucidated.

Other modes of action were evaluated in Appendix 1 and in conclusion there is no compelling evidence for any other MOA for the developmental toxicity of flumioxazin in the rat.

3. Plausibility of mechanism at low doses (30 mg/kg/day) in the rat

3.1 Critical period of sensitivity and pharmacokinetics

Single oral treatments of flumioxazin at 400 mg/kg were given to pregnant rats on different gestational days (Kawamura, 1993; SBT-30-0044). Results showed that GD 12 is the day of greatest sensitivity to the developmental effects of flumioxazin including fetal death, reduced fetal bodyweight and VSD (Table 1).

Table 1: Summary results of the sensitive period-finding study with flumioxazin

Gestation Day	Dose (mg/kg)	Embryonic Death (%)	Fetal 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 and the effect levels were identical at 30 mg/kg in successive administrations and at 400 mg/kg in single doses during the sensitive period. The evidence supports the identical mode of action to be the basis of the three types of developmental toxicities between the developmental toxicity study at 30 mg/kg and the sensitive period-finding study at 400 mg/kg.

It is not uncommon that a single higher dose is necessary to produce the developmental toxicity which is similar to those produced by multiple doses. In the case of finasteride, developmental toxicity produced by repeated doses of 3 mg/kg is comparable to two doses of 20 mg/kg, which is 7 times higher than 3 mg/kg (Table 2) (Clark *et al*., 1990; Clark *et al*., 1993).

Table 2: Comparison of doses of finasteride which produce abnormalities in foetuses between single and multiple doses

Dosing type	Dosing period	Dose (mg/kg/d)	Hypospadias (%)
multiple	2wks prior mating to GD20	3	ca.35
twice	GD16-17 (critical period)	20	19

Considering a decrease in absorption rate with increment in oral dose, difference in internal dose levels would not be as large as those calculated from dose levels orally administered. The internal dose of 400 mg/kg is 84 mg/kg and becomes 5.6 times higher than that of 30 mg/kg, which is 15 mg/kg. As a result of calculation with the physiologically-based pharmacokinetic (PBPK) model, Cmax and AUC of 400 mg/kg are 4 times and 6 times larger than that of 30 mg/kg (Table 3).

A single treatment at 400 mg/kg could be comparable to a repeated treatment at 30 mg/kg during the sensitive period.

Table 3: Internal concentrations of flumioxazin at different oral dose levels

	Cmax (µg/mL)	AUC (µg x hr/mL)	Internal dose (mg/kg)
Fetus (30 mg/kg po single dose, actual)	0.06	0.72	15
Fetus (30 mg/kg po single dose, simulated)	0.06	1.05	15
Fetus (30 mg/kg po repeat 9 daily doses)	0.08	-	-

	Cmax (µg/mL)	AUC (µg x hr/mL)	Internal dose (mg/kg)
Fetus (400 mg/kg po single dose, simulated)	0.24	5.99	84
Fetus (1000 mg/kg po single dose, simulated)	0.32	8.68	120

Cmax, AUC and internal dose was predicted by the PBPK model of flumioxazin concentration in the fetus of pregnant rats orally dosed with flumioxazin at a single dose of 30, 400, and 1000 mg/kg po (Takaku 2012(c); SBM-0093). Adjustment of absorption at doses of 30, 400, and 1000 mg/kg, are 50% (actual value), 21% (logarithmic approximation), and 12% (actual value), respectively (Takaku 2012a, SBM-0089; Takaku 2012b, SBM-0092 and Takaku 2012c: SBM-0093).

The small amount of radioactivity in the rat fetus vs adult, *i.e*. total radio activities were detected at concentration of 3.34 and 1.14 µg eq./g in maternal plasma and fetus, respectively (Shirai 2009, SBM-0081). However, flumioxazin was detected at a concentration of 0.02 µg eq./g in both maternal plasma and fetus (Table 4). Therefore, there was no difference to exposure to flumioxazin between maternal animals and fetuses.

Table 4. Concentration of flumioxazin and its metabolites in the tissues of pregnant rats at 7 hours after administration

Compound	Concentration (µg eq./g or mL)	
	Maternal Plasma	Fetus
Total radio activity	3.34	1.14
Flumioxazin	**0.02**	**0.02**
APF	0.94	0.48
3-OH-S-53482	0.08	0.01
4-OH-S-53482	0.02	0.01
^{14}C-3-OH-S-53482-SA	0.03	0.01
^{14}C-4-OH-S-53482-SA	0.02	0.01
^{14}C-Ac-APFA	0.13	0.02
Other	2.30	0.58

3.2 PPO inhibition and PPIX accumulation in the fetus vs adults

PPIX accumulation in embryos corresponded to flumioxazin developmental toxicity in terms of species difference, compound-specific difference, and peak sensitivity (Kawamura, 1997; SBT-0063). This correlation demonstrates a close link between PPO inhibition and developmental abnormality (Figure 3).

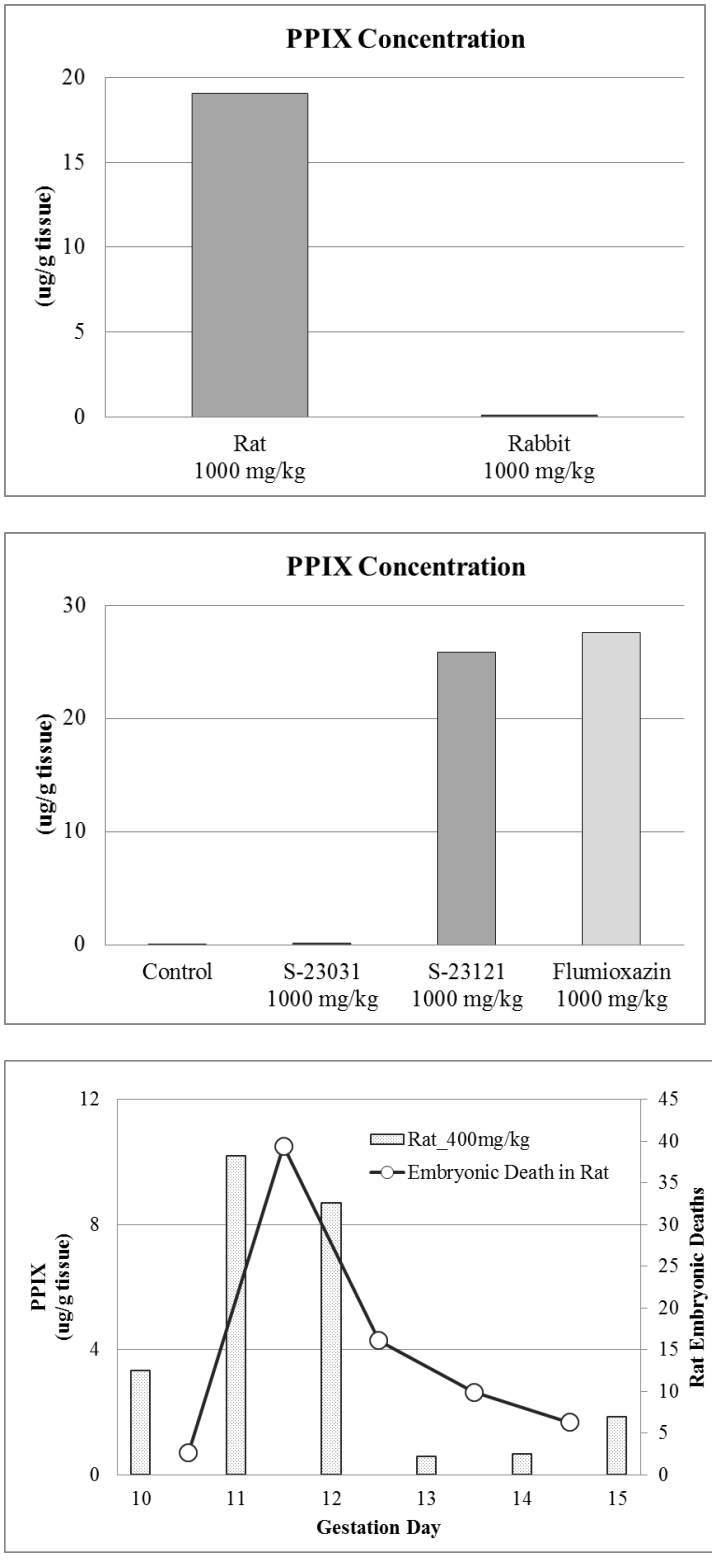

Fig. 3 Close link between PPIX accumulation and developmental toxicity

Concentrations of PPIX in maternal liver are lower than peak concentrations in embryo, as shown in Table 5. This could be due to rapid excretion into bile and feces in the adult (Krut J. et al., 1992).

Table 5: PPIX concentration in rat embryos and maternal liver samples (report SBT-0063)

Parameter		Gestation day of administration					
		10	11	12	13	14	15
Flumioxazin (400 mg/kg)	PPIX conc. /embryo (µg/g tissue ± SD)	3.335 ± 1.204	10.214 ± 7.007	8.701 ± 5.837	0.610 ± 0.249	0.684 ± 0.497	1.853 ± 1.229
	PPIX conc. /maternal liver (µg/g tissue ± SD)	0.245 ± 0.072	0.364 ± 0.246	0.359 ± 0.090	0.220 ± 0.048	0.287 ± 0.051	0.275 ± 0.034

Species- and compound-related differences were observed in *in vitro* inhibition of PPO (Table 6) and *in vitro* inhibition corresponded closely with PPIX accumulation and teratogenicity. Sensitivity of PPO activity extracted from adult female liver was found to be comparable to that of embryonic PPO, suggesting that inhibition of adult liver PPO is indicative of embryonic PPO inhibition (Green 1993, SBT-31-0045; Dabbs 1996, SBT-0060).

Table 6: Comparison of IC_{50s} (nM) for PPOs derived from adult rat and rabbit liver and embryo

	Flumioxazin		S-23121		S-23031	
	Rat	Rabbit	Rat	Rabbit	Rat	Rabbit
Adult liver	8	52	11	1560	793	4750
Embryo day12	12	95	47	6490	344	5920
Embryo day15	6	308	20	1270	204	5090

3.3 PPIX accumulation and reduced haem biosynthesis in rat REL cells

To experimentally demonstrate that rat erythroid cells are sensitive to the disturbance of haem synthesis and induction of anaemia by flumioxazin-induced PPO inhibition, we conducted studies with REL cells (cell line, derived from transplantable tumors from 7,12 dimethylbenz (a) anthracene-induced erythroleukemia in the Long-Evans rat (Kluge, 1976) . In rat REL cells, haem biosynthesis was reduced at the concentration of 0.1µM, which is close to that in embryos whose mother was exposed to 30 mg/kg flumioxazin (Table 7) (Kawamura 2013). Reduced haem biosynthesis and accumulated PPIX were simultaneously observed at 0.1 µM and above, indicating reduced PPO activity was becoming rate-limiting.

Table 7: PPIX and haem concentrations in REL cells

Dose of flumioxazin	Embryonic tissue concentration (Predicted by PBPK) (1)	Experimental concentr.	Hepatocyte (Rat) (2)	REL cell line (Rat) (3)	
			PPIX	PPIX	Haem
mg/kg bw*	µM	µM	pg /mg protein (%Control)	ng/10^6cells (%Control)	ng/10^6cells (%Control)
0	0	0	**293**	**0.63**	**127**
		0.01		**0.60 (95)**	**116 (91)**
		0.03	**369 (126)**		
		0.08	**524 (179)**		
		0.1		**1.11 (176)**	**91 (72)**
30 single	0.17				
		0.28	**1200 (410)**		
		0.3		**1.94 (308)**	**85 (67)**
400	0.68				
		0.84	**3000 (1024)**		
1000	0.90				
		1.0		**5.95 (944)**	**60 (47)**
>1000		5.0		**14.0 (2222)**	**47 (37)**

These results demonstrate the sensitivity of the rat erythroid cell to reduced haem synthesis at a concentration similar to that from repeated doses of flumioxazin at 30 mg/kg.

(1) Embryonic tissue concentrations are predicted by PBPK model described on Takaku (2012c).

(2) Abe 2011, SBT-0120

(3) Kawamura 2013, SBT-0125

3.4 Maternal vs fetal anaemia at 30 mg/kg/day

Manifestation of anaemia caused by flumioxazin is not identical between adults and embryos in rats even though PPO inhibition is comparable between adults and embryos. This is because rat embryos are susceptible to flumioxazin exposure within a shorter period of time than adults due to a single wave of synchronous maturation of rat primitive erythroblasts in yolk sac. In adults, continuing hematopoiesis and remaining erythroblasts non-sensitive to flumioxazin can compensate for effects of flumioxazin on sensitive erythroblasts during a period of compensable time. In adults flumioxazin is necessary to affect beyond compensable time period due to continuous waves of hemopoiesis. Also fetuses are less sensitive to flumioxazin once passing the peak sensitive period since a major site of hematopoiesis sifts to the liver and continuing hematopoiesis occurs. Thus, the fetus would develop severe anaemia during the sensitive period in the absence of significant anaemia in the adult during several days of exposures to flumioxazin.

4. Conclusion on plausibility of the mechanism

There is convincing evidence for a single mode of action causing the developmental toxicities in rats. The sequence of key biological events in the proposed mode of action has been elucidated.

Observed types and incidences of developmental toxicity by flumioxazin are identical between repeated doses of 30 mg/kg and a single dose of 400 mg/kg. Considering a decrease in absorption rate with increment in oral dose, difference in internal dose levels would not be as large as those calculated from dose levels orally administered. The evidence supports the identical mode of action to be the basis of developmental toxicities between the developmental toxicity study at 30 mg/kg and the sensitive period-finding study at 400 mg/kg.

Rat embryos are susceptible to flumioxazin exposure within a shorter period of time than adults due to a single wave of synchronous maturation of rat primitive erythroblasts in yolk sac during the sensitive period while PPO inhibition is comparable between adults and embryos. In adults, continuing hematopoiesis and remaining erythroblasts non-sensitive to flumioxazin can compensate for effects of flumioxazin on sensitive erythroblasts during a period of compensable time. Thus, the fetus would develop severe anaemia during the sensitive period in the absence of significant anaemia in the adult during several days of exposures to flumioxazin.

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

Appendix 1 Evaluation of other potential Modes of Action

1. Accumulated PPIX

Because PPIX accumulation corresponded to the developmental toxicity of flumioxazin, it might be assumed that developmental toxicity of flumioxazin is mediated through the same mode of action as the herbicidal one. If photodynamic action is a cause of developmental toxicity, a photodynamic dye should be a developmental toxicant. However a photodynamic dye, rose bengal, exhibited neither embryolethality nor teratogenicity in rats (2). The light would not sufficiently reach embryos through the maternal body wall to induce photodynamic action of accumulated PPIX in embryos.

Protoporphyrin IX is assumed to be an endogenous ligand to the peripheral benzodiazepine receptor on mitochondria. Presumably acting through the receptor, PPIX suppressed DNA replication in mouse spleen lymphocytes *in vitro* (3). Nevertheless many of the benzodiazepoines such as diazepam, lorazepam, clonazepam, and oxazepam failed to exhibit teratogenicity in rats (4). Accumulated PPIX is considered to be indicative of PPO inhibition rather than a causative factor in teratogenicity.

2. Form of anaemia

Flumioxazin caused hypochromic microcytic anaemia that generally occurs as a consequence of impaired hemoglobin synthesis (5). Impairment of hemoglobin synthesis is caused by iron-deficiency or defective porphyrin metabolism resulting in abnormal iron accumulation in erythroblasts termed sideroblasts. As indicated in mechanistic studies, iron deposition in mitochondria was an initial histological change and increased sideroblasts were observed. Flumioxazin induced anaemia is due to inhibition of porphyrin metabolism rather than iron deficiency.

3. Relationship between fetal death and malformation

In some cases fetal death is attributable to malformation. Beck and Lloyde investigated the relationship between fetal deaths and fetal malformation by treating rats with trypan blue at day 8.5 and examining the uteri and fetuses on days 11.5, 14.5 and 20.5 of gestation (6). Because the incidence of fetal malformations fell with a corresponding rise in fetal deaths as pregnancy proceeded, the authors concluded that fetal death was a result of pre-existing fetal malformation in the majority of cases. In the flumioxazin studies, most of the dead fetuses were observed by day 15 while VSD can be diagnosed following completion of closure of the interventricular foramen on day 16. Consequently, fetuses were dead prior to closure of the interventricular septum indicating that VSD is not the direct cause of conceptal death but rather occurs in some surviving fetuses. This supports fetal anaemia as the cause of embryonic deaths rather than deaths from malformations.

4. VSD

Initial histological changes observed in rat embryos were iron deposits in mitochondria and dilatation of mitochondrial matrix space in polychromatophilic erythroblasts. Following the mitochondrial lesions, affected erythroblasts degenerated in the embryonic circulation and were engulfed by macrophages. Treatment-related changes in the embryonic cardiovascular system and liver accompanied the appearance of erythroblastic lesions. Thinning of the ventricular walls of the heart is indicative of dilatation of the ventricles, and reflects a compensatory reaction to embryonic anaemia since enlargement of heart was observed corresponding to decreased hemoglobin content and reduced red blood cell number. Increased stroke volume in the heart is an important reaction to anaemia (7). No treatment-related changes in myocardial cells were observed at the electron

microscopic level. As noted previously, the interventricular foramen closes from day 15 to day 16. In our studies, exposed hearts were enlarged from day 14 and completion of ventricular septa formation was delayed.

Clark has proposed five pathogenic modes of actions for some congenital cardiac malformations based on mechanism rather than anatomic anomaly. They are ectomesencymal tissue migration abnormalities, abnormal intracardiac blood flow (cardiac hemodynamics), cell death, extracellular matrix, and abnormal targeted growth (8). Clark stated that perimembranous ventricular septal defect may represent abnormal fusion of the muscular, inflow and outflow septa, and that deviation of the septal components by abnormal blood flow pattern may lead to defects in this region of the heart (9).

A comparison of sensitive periods for development of VSD between flumioxazin and several other agents shows considerable differences in peak sensitivity. X-ray irradiation (10) and nimustine (11), an alkylating agent, or bisdiamnie (12), which acts on the proliferation or migration of mesencyme, probably produce VSD by direct injurious effects (cell damage) on the fetal heart. The peak of sensitivity to these agents occurs between days 8 – 10, while the most sensitive day for flumioxazin-induced-VSD is gestational day 12.

Earlier studies by Haring (13) and Clemmer and Telford (14) support the proposed mechanism by showing that prenatal hypoxia produces cardiovascular abnormalities including VSD in rats. Jaffee stated that, when hypoxia was used as a teratogenic agent following the onset of circulation, distortion of the form of the heart tube was a primary lesion (15).

Thus, overall it is concluded that VSD caused by flumioxazin is attributed to fetal anaemia and not to any other direct injurious effect on the heart.

5. Wavy ribs

Wavy ribs are induced in the later stages of rib chondrification and ossification, and may be indicative of fetal pathology as opposed to malformations. It may be possible that many agents produce wavy ribs through several mechanisms leading to two final common effects including inhibition of mineralization and increased uterine tone. Renal loop diuretics and beta-stimulants have been studied in detail because they are associated with a high incidence of wavy ribs. Maternal serum chloride was decreased after treatment with furosemide, a renal loop diuretics. Co-administration of a muscular relaxant reduced the incidence of wavy ribs after furosemide exposure. Decreased fetal serum alkaline phosphatase and total protein were reported following exposure of fenoterol, a beta-stimulant (16). Flumioxazin decreased fetal serum protein and increased incomplete/delayed ossification of the ribs. The increased incidence of wavy ribs is more likely to be associated with these changes rather than being caused by a different mechanism.

6. Link between fetal anaemia and developmental toxicity

A link between fetal anaemia and developmental effects observed in the flumioxazin teratogenicity study is also demonstrated in recent studies with artesunate, an anti-malarial drug. Artesunate induces developmental abnormalities consisting of fetal death, growth retardation and anomalies such as VSD, rib abnormalities and bent long bones (17). Embryonic erythroblasts are the primary target of artesunate toxicity and consequent embryonic anaemia resulted in developmental toxicity similar to that produced by flumioxazin (18).

7. Species difference in metabolism between rats and rabbits

When pregnant rats and rabbits received oral administration of ^{14}C-flumioxazin at 30 mg/kg for seven consecutive days, no clear pattern of absorption, distribution, metabolism or excretion was seen that could account for the species specific developmental toxicity in rats. After initial dose, Cmax/min of ^{14}C concentration in plasma ranged from 4.49 to 0.70 in rats and from 4.14 to 1.02 in rabbits. In both species most of the previous dose of ^{14}C was excreted before the next dose, and the metabolic profiles of flumioxazin were similar (19).

8. PPO inhibitory activity of metabolites

Oral doses of [phenyl-^{14}C] flumioxazin (30 mg/kg) administered to pregnant rats from gestational days 6 through 12 cross the placenta and reach the rat fetus. Major metabolites in the fetus included 3OH-flumioxazin, 4OH-flumioxazin, and APF (19). In order to determine the active form that inhibits PPO, we employed *in vitro* PPO inhibition assays using liver extracts prepared from adult, female livers. Experiments were conducted with flumioxazin and its three major metabolites (20). The results showed that flumioxazin was the strongest PPO inhibitor. There was no metabolite that could account for the species-specific developmental toxicity in rats based on the degree of PPO inhibition.

The possibility of a direct effect of metabolites on developmental toxicity is also considered. APF was detected at higher concentrations in rat fetuses compared with other metabolites. It is a benzoxazinone moiety formed from cleavage of the amide linkage. There is convincing evidence that embryolethality and VSD are attributed to the consequences of fetal anaemia and that the fetal malformations are not the causative factors in embryonic deaths. The spectrum of developmental effects associated with flumioxazin is consistent with a single mode of action. Therefore, it is considered very unlikely that a metabolite would be a direct acting teratogen causing VSD and skeletal anomalies by a mechanism that was independent of fetal anaemia and embryolethality. Furthermore, the main metabolite APF was also detected in rabbit fetuses and no developmental toxicity was seen in rabbits at a dose level 100 times higher than that causing developmental toxicity in the rat. Fetal concentrations of metabolites at this dose level in the rabbit would be much higher than those in the rat.

Conclusion:

A peak sensitive period was common to embryolethality, teratogenicity and growth retardation with flumioxazin and the effect levels were identical at 30 mg/kg in successive administrations and at 400 mg/kg in single doses during the sensitive period. The evidence supports a single mode of action to be the basis of the three types of developmental toxicities.

In conclusion there is no compelling evidence for any other MOA for the developmental toxicity of flumioxazin in the rat.

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ANNEX 2
ASSESSMENT OF THE HUMAN RELEVANCE OF THE DEVELOPMENTAL EFFECTS INDUCED BY FLUMIOXAZIN IN RATS

2. Assessment of the human relevance of the developmental effects induced by flumioxazin in rats

Substance 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

Dossier submitter: Ministry of Environment of the Czech Republic

Department of Environmental Risks and Ecological Damages

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Czech Republic

Prepared by Sumitomo Chemical Co., Ltd

2014. 3. 31

Assessment of the human relevance of the developmental effects induced by flumioxazin in rats

Executive summary

This document has been prepared to address the points raised in the RAC Opinion Development Document (ODD) and those arising at the RAC meeting (13/03/2014). The information presented in this document focuses on the areas of uncertainty in the ODD relating to the human relevance of the mechanism of developmental effects induced by flumioxazin in the rat.

The following conclusions are drawn based on the information presented.

1. The *in vitro* rat REL erythroid cell is an appropriate model for assessing the effects on erythroids *in vivo*

2. Reduced heme biosynthesis is observed in REL cells at a concentration of 0.1 µM, close to that in embryos whose mother was exposed to 30 mg/kg flumioxazin and where PPIX accumulation is observed.

3. In contrast, in human fetal cells (CD36+ derived from cord blood) or human K562 cells, heme biosynthesis is not reduced at concentrations up to 5 µM where PPIX accumulates. The *in vitro* human cells are considered appropriate models for predicting the effects on erythroids *in vivo* in the human adult and fetus.

4. PPO activity could be close to that of the rate-limiting enzyme and reduced PPO activity becomes rate-limiting in rats, but not in humans. The overall difference in inhibitory activity of heme biosynthesis between rats and humans is greater than 50 times (0.1 µM vs 5µM).

5. The PBPK model is shown to be a justifiable approach to predict flumioxazin concentrations in the fetus of the pregnant human. The model shows that non susceptible concentrations of up to 5 µM in human erythroblastswould far exceed those attained in human embryos following flumioxazin exposure at a dose of 1000 mg/kg.

In conclusion, it is highly unlikely that humans are susceptible to the fetal anaemia and developmental effects of flumioxazin as seen in rats owing to the substantial qualitative and quantitative species differences between the rat and human.

ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON FLUMIOXAZIN (ISO); N-(7-FLUORO-3,4-DIHYDRO-3-OXO-4-PROP-2-YNYL-2H-1,4-BENZOXAZIN-6-YL)CYCLOHEX-1-ENE-1,2-DICARBOXIMIDE

Contents

ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON FLUMIOXAZIN (ISO); N-(7-FLUORO-3,4-DIHYDRO-3-OXO-4-PROP-2-YNYL-2H-1,4-BENZOXAZIN-6-YL)CYCLOHEX-1-ENE-1,2-DICARBOXIMIDE

1. Introduction

This document has been prepared to address the points raised in the RAC Opinion Development Document (ODD) and those arising at the RAC meeting (13/03/2014). The additional information presented in this document focuses on the areas of uncertainty in the ODD relating to the human relevance of the mechanism of developmental effects induced by flumioxazin in the rat, reproduced below:

***B. Human relevance**:*

The human relevance of the mechanism is questioned (by the DS) on the basis that:

-A clear species difference is demonstrated between rats and rabbits with respect to the developmental toxicity induced by flumioxazin. This is supported by the lower sensitivity to PPO inhibition and PPIX accumulation in rabbits.

- The difference between rats and human with respect to PPO inhibition is less marked e.g., IC50 measured in liver mitochondria; 0.007, 0,017, 0.138, in rat, human and rabbit, respectively). The species difference in PPIX induction shown in primary hepatocytes was 10.1, 4.4, 1.1, and 1.4 fold in rat, human, monkey and rabbit, respectively. The reason for the different species sensitivity to flumioxazin induced PPO inhibition is not known.

-It is proposed that human foetus will be less sensitive to anaemia induced erythroblast damage than the rat due to the synchronous maturation of the erythroblasts and the sensitivity of polychromatophilic erythroblasts on a single day (12) of gestation in rats.

-It was demonstrated that human erythroid cell lines (K562/CA36+) could differentiate normally in the presence of flumioxazin and that haem production was not affected even though PPO inhibition occurred. PPO inhibition was associated with reduced haem in a rat REL cell line.

***However**, the demonstrated difference (PPO inhibition 2.4 –difference) between rat and human cells is not great and represents a moderate quantitative difference.*

-Even though rat embryos may be particularly sensitive to anaemia induced by distruction of polychromatophilic erythroblasts in day 12, it is not known what the effect may be in human embryos during the sensitive period of erythroblast maturation.

Note: The antimalarial artemisinins target embryonic erythroblasts and maternal reticulocytes (via damage to haem producing mitochrondia) causing embryotoxicity and maternal reticulocytopenia in rats. The authors conclude that the therapeutic dose range causing maternal reticulocytopenia in pregnant women is associated with a risk of adverse effects on the embryo. (Clark 2011). These authors have previously reported developmental toxicity and teratogenicity of artemisinins in rats at doses causing only a 15% reduction of maternal reticulocytes, ie., embryos are more sensitive and also concluded in the previous paper (2008) that ..'doses in (human) pregnancy during the sensitive period (post conception day 21 to week 9) which might cause even a minor decrease in adult reticulocyte count could cause a marked depletion of embryonic erythroblasts which could lead to death or malformation of the embryo'.

The mechanism of toxicity is not the same for flumioxazin but the target cells appear to be, so the point about sensitivity of both rat and human embryonic erythroblasts is relevant.

2. Relative sensitivity to inhibition of PPO by flumioxazin among species

Since PPO inhibition is the initial events in the developmental toxicity of flumioxazin, species differences in PPO inhibition *in vitro* (liver) in rats and humans were investigated. Rat PPO was most sensitive to inhibition by flumioxazin among the species tested while humans were intermediate between rats and rabbits (Table 1).

Table 1 Comparison of IC_{50s} (nM) among three species for PPOs derived from adult liver

Species	rat	rabbit	human
IC_{50}	7.15	138	17.3

PPO exists in complicated and highly-organized structure in mitochondria. Accessibility of flumioxazin to PPO in the cell can be different from that in the *in vitro* model employing mitochondrial extracts. Therefore, PPO inhibition by flumioxazin was investigated using cryopreserved hepatocytes of rats, rabbits, monkeys and humans (Abe, 2011). The results are shown in Fig. 1. No PPIX accumulation was observed in rabbit and monkey hepatocytes at the maximum tested concentration of flumioxazin. Remarkable accumulation of PPIX as the result of PPO inhibition in hepatocytes was observed in rats with a smaller amount of PPIX detected in human hepatocytes. These results show that human PPO is less sensitive to flumioxazin than rat PPO even at the cellular level, reflecting *in vitro* inhibitory potency of flumioxazin.

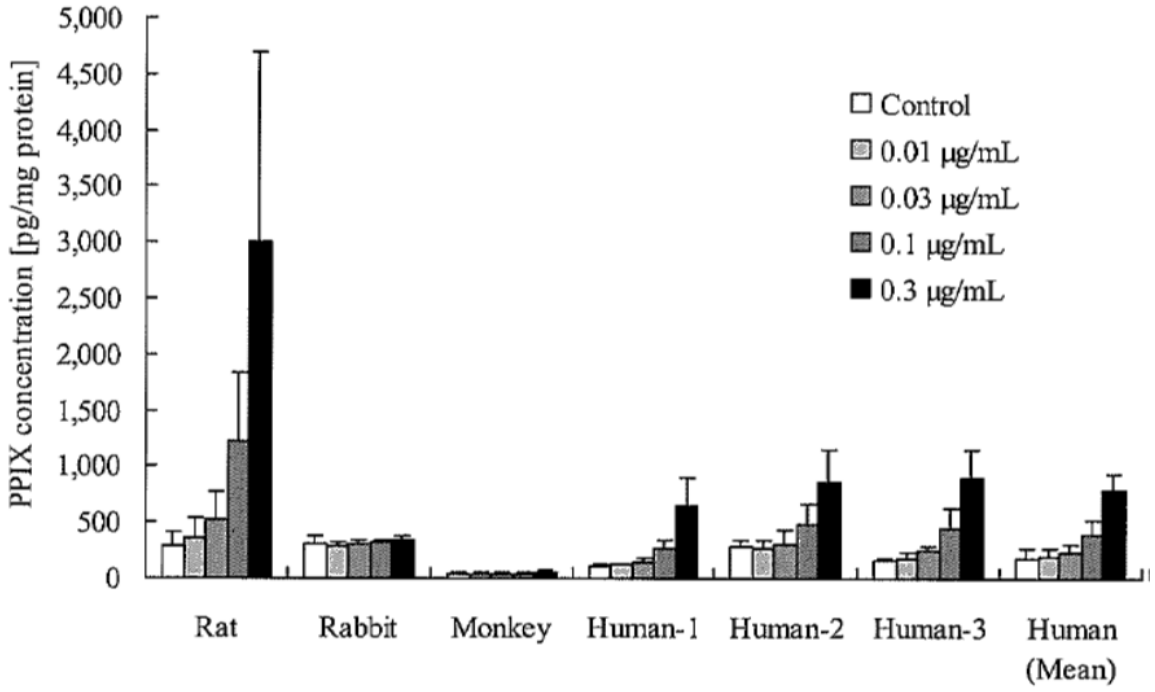

Fig. 1 PPIX accumulation in cryopreserved hepatocytes induced by flumioxazin

The factor of the fact that PPO deficient patients do not include anemia or hematological problem as described later, demonstrates that human would be more resistant to induction of anemia by PPO inhibition than rats. Sumitomo further investigated other species difference between rats and humans.

3. Induction of anemia by PPO inhibition

In general toxicity studies and teratogenicity studies of flumioxazin, the rat is the most sensitive animal species among rats, mice, dogs and rabbits. Anemia, attributable to PPO inhibition, is the primary toxic effect in rats caused both in adults and embryos.

Sumitomo has investigated whether or not PPO inhibition in erythroblasts can cause anemia in humans. To experimentally demonstrate that human erythroids are resistant to the disturbance of heme synthesis and induction of anemia by flumioxazin-induced PPO inhibition, Sumitomo conducted studies with K562 (Kawamura, 2011), CD36+ (Kawamura, 2013a), and REL cells (Kawamura, 2013b). Their origins are summarized in Table 2. They are used as a model for human and rat erythroid maturation since the cells can be differentiated into hemoglobin-synthesizing cells.

Table 2 Origin of K562, CD36+ and REL cells

Cell type	Origin
CD36+ cell	Human CD36+ cells were derived from human fetal cord blood
K562 cell	Cell line, derived from a patient with chronic myeloid leukemia in the acute phase[1]
REL cell	Cell line, derived from transplantable tumors from 7,12 dimethylbenz (a) anthracene-induced erythroleukemia in the Long-Evans rat (Kanoh, 1982)

1) CB Lozzio, BB Lozzio. (1975) Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. Blood. 45: 321-334.

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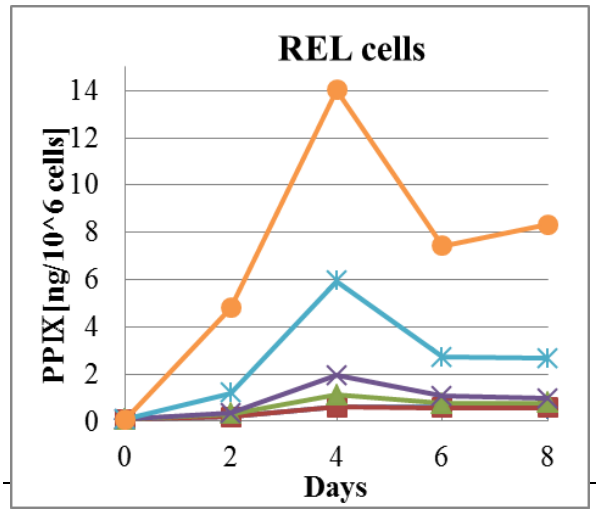
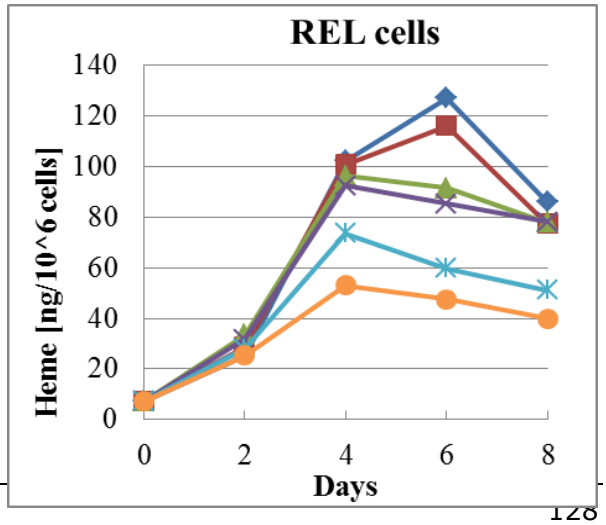
Although accumulation of PPIX resulting from PPO inhibition was observed, no effects were observed on heme content and cell proliferation in human hemoglobin-synthesizing K562 and human fetal CD36+ cells (Fig. 2, table 3) (Kawamura, 2011) even when treated with 5 µM of flumioxazin. This concentration is close to its water solubility limit (1.79 mg/L) (Saito, 1991). It is also many times greater than the concentrations 0.06 ppm (0.17 µM) or 0.02 ppm (0.056 µM) in rat embryos from dams treated singly or repeatedly with 30 mg/kg predicted by PBPK model described by Takaku (2012a). In contrast, in rat REL cells, heme biosynthesis was reduced at the concentration of 0.1µM, which is close to that in embryos whose mother was exposed to 30 mg/kg flumioxazin. Reduced heme biosynthesis and accumulated PPIX was simultaneously observed at 0.1 µM and above in REL cells, indicating reduced PPO activity becomes rate-limiting in the rat but not in human.





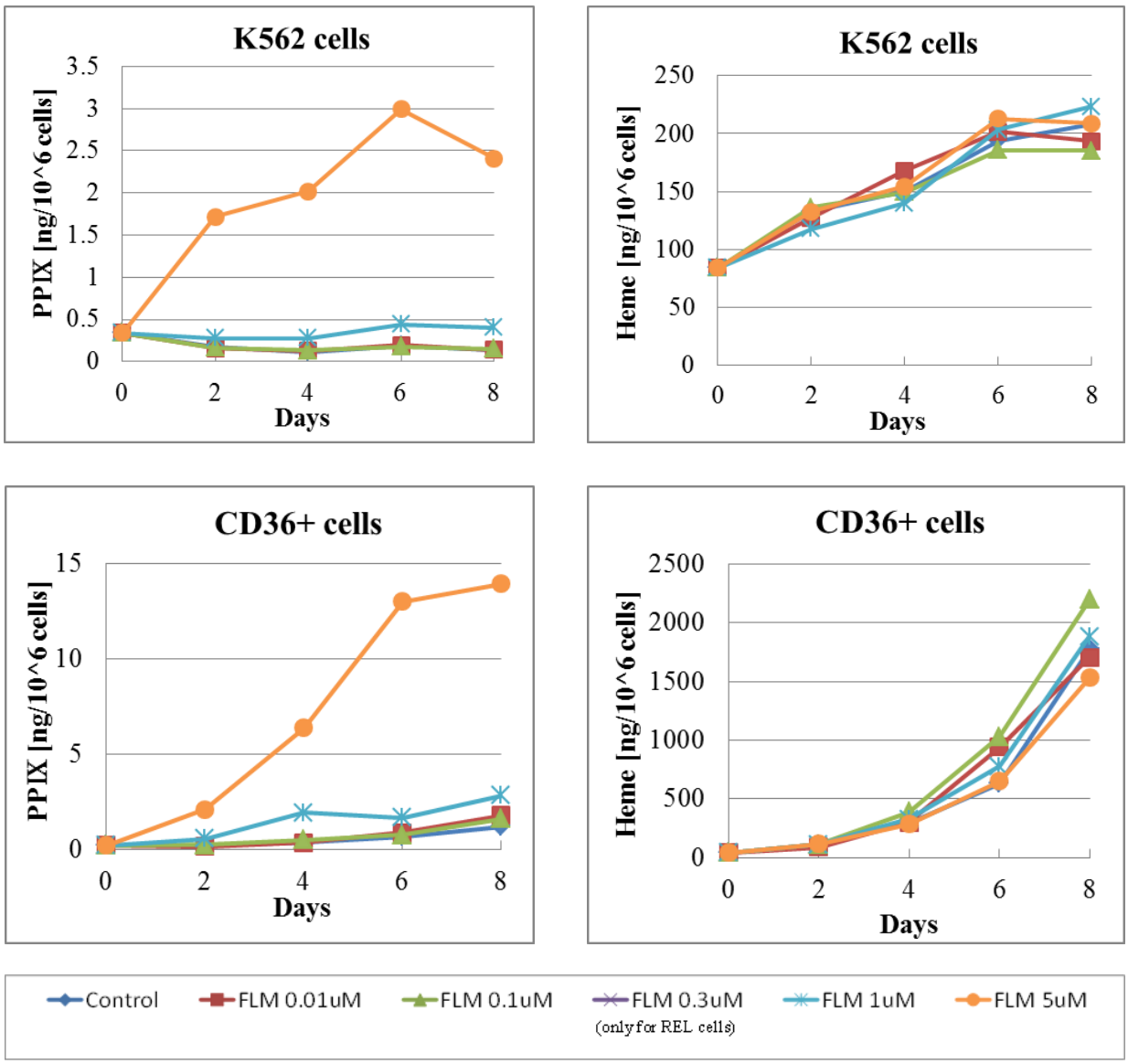


Fig.2 Effects of flumioxazin on PPIX accumulation and heme biosynthesis in K562, CD36+ and REL cells

Table 3: PPIX concentrations in liver and PPIX and haem concentrations in K562, CD36+ and REL cells

Dose of flumioxazin	Embryonic tissue concentr. (predicted by PBPK) (1)	Experi-mental conc.	Hepatocyte (Rat) (2)	Hepatocyte (Human) (2)	REL cell line (Rat) (3)		K562 cell line (Human) (4)		CD36+ cell (Human) (5)	
			PPIX	PPIX	PPIX	Haem	PPIX	Haem	PPIX	Haem
mg/kg bw *	µM	µM	pg /mg protein (%Control)	pg /mg protein (%Control)	ng/ 10^6cells (%Control)	ng/10^6cells (%Control)	ng/ 10^6cells (%Control)	ng/ 10^6 cells (%Control)	ng/ 10^6 cells (%Control)	ng/ 10^6cells (%Control)
0		0	**293**	**180**	**0.63**	**127**	**0.19**	**208**	**1.18**	**1777**
		0.01			**0.60(95)**	**116(91)**	**0.19(100)**	**194(93)**	**1.77(150)**	**1706(96)**
		0.03	**369 (126)**	**190 (106)**						
		0.08	**524 (179)**	**233 (129)**						
		0.1			**1.11(176)**	**91(72)**	**0.18(95)**	**185(89)**	**1.59(135)**	**2198(124)**
30 single	0.17									
		0.28	**1231 (420)**	**404 (224)**						
		0.3			**1.94(308)**	**85(67)**				
400	0.68									
		0.84	**3007 (1026)**	**799 (444)**						
		1.0			**5.95(944)**	**60(47)**	**0.44(232)**	**224(108)**	**2.82(239)**	**1882(106)**
1000	1.9 (Human)									
>1000		5.0			**14.0(2222)**	**47(37)**	**3.0(1579)**	**209(100)**	**14.0(1186)**	**1535(86)**

(1) Embryonic tissue concentrations are predicted by PBPK model described by Takaku (2012a).

(2) Abe 2011

(3) Kawamura 2013b

(4) Kawamura 2011

(5) Kawamura 2013a

Overall difference in inhibitory activity of heme biosynthesis between rats and humans is greater than 50 times (0.1 µM vs 5µM). The validity of extrapolating the *in vitro* results on human erythroid cells to the fetus is supported by the data on artemisinins where inhibition of haemoglobin biosynthesis in human K562 cells correlates with embryonic anaemia amongst a range of animal species, including monkeys (see point 6 below). There is no species difference in response, unlike flumioxazin. Thus the absence of any effect of flumioxazin on haem. biosynthesis in K562 cells and CD36+ cells is predictive of the human fetus *in vivo*.

4. Prediction of flumioxazin concentrations in human embryos

To investigate the significance of the concentration of 5 µM in assessing the developmental toxicity of flumioxazin in humans, a physiologically based pharmacokinetic (PBPK) model for flumioxazin was developed to predict flumioxazin concentration in the maternal blood and fetus of pregnant

human. A PBPK model is recommended to clarify the difference of pharmacokinetics between animals and humans in the guideline of OECD (OECD 2008) and guidance from the IPCS (IPCS 2010) and ECHA (ECHA 2012). Flumioxazin concentrations in pregnant rats (30 mg/kg po) were used to develop the PBPK model in pregnant rats using physiological parameters from the literature and chemical-specific parameters from our experimental results and fitting, then, the developed rat PBPK model was extrapolated to the human model. An *in vitro* metabolism study using rat and human liver microsomes was conducted to analyze 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 % absorption of flumioxazin after oral administration at 1000 mg/kg (Takaku 2012b). 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 as in rats. The developed human pregnant model demonstrated that flumioxazin concentration in the human fetus at dose of 1000 mg/kg po was 0.68 ppm (1.92µM) (figure 3) (Takaku 2012c). This concentration is lower than the maximum no effect concentration of 5 µM in human K562 cells and CD36+ cells supporting the view that humans would not be susceptible to anemia and the developmental effects of flumioxazin..

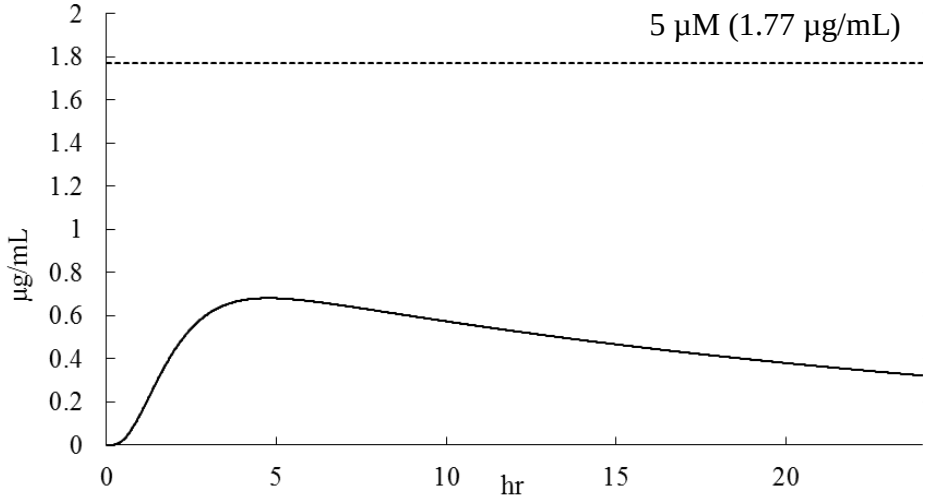


Fig 3: Calculated flumioxazin concentration in the fetus of pregnant humans orally dosed with flumioxazin at a dose of 1000 mg/kg

Calculated flumioxazin concentration in the fetus of pregnant humans orally dosed with flumioxazin at a dose of 1000 mg/kg (solid line) and dashed line of 5 µM (1.77 µM) by developed physiologically based pharmacokinetic (PBPK) model (Takaku 2012a).

Human erythroblasts are concluded to be non-susceptible to flumioxazin when treated at concentrations as high as 5 µM. These concentrations are expected to far exceed those attained in human embryos following flumioxazin exposure.

Experimental data demonstrate that PPO activity in human erythroids would remain higher than the activity of a rate-limiting enzyme in the heme synthetic pathway, and that humans would not become anemic as a result of flumioxazin exposure even in the presence of PPIX accumulation caused by PPO inhibition.

5. Additional factors leading to less sensitivity of humans to flumioxazin

5.1 Synchronous maturation of erythroblasts

In rats, a characteristic of hemopoiesis in yolk sac is that erythroid cells undergo synchronous maturation as a relatively homogeneous population. The morphology and population characteristics of blood cells in rat embryos demonstrated that a vast majority of erythroblasts are polychromatophylic on gestational day 12, the day of the greatest sensitivity, and orthochromatophilic erythroblasts on gestational day 14, when rat embryos were much less sensitive to flumioxazin (Ihara 2011).

This explains, in part, why flumioxazin induces an enormous and synchronous loss of blood cells in rat embryos exposed to flumioxazin. In contrast to rats, a relatively heterogeneous population was observed in human primitive hemopoiesis by Kelemen (Fig. 4) (Kelemen 1979a), who classified the erythroblast into three types. It is conceivable that the type III erythroblast corresponds to the orthochromatophilic erythroblast, and type I and type II correspond to earlier erythroblasts, presumably basophilic or polychromatophilic. Relative populations of type I, II, and III observed in yolk sac range from 7% to 40%, from 21% to 89%, and from 4% to 65%, respectively, during the period from commencement of human primitive hemopoiesis in week 3-4 to completion of ventricular septum formation in week 8 (Kelemen 1979b). Thus in humans, even if a particular population is lost, blood cell loss could not be as massive as in rats.

As described above, human fetal erythroblasts (from cord blood) are non-susceptible to flumioxazin when treated at concentrations as high as 5 µM. These concentrations are expected to far exceed those attained in human embryos following flumioxazin exposure.

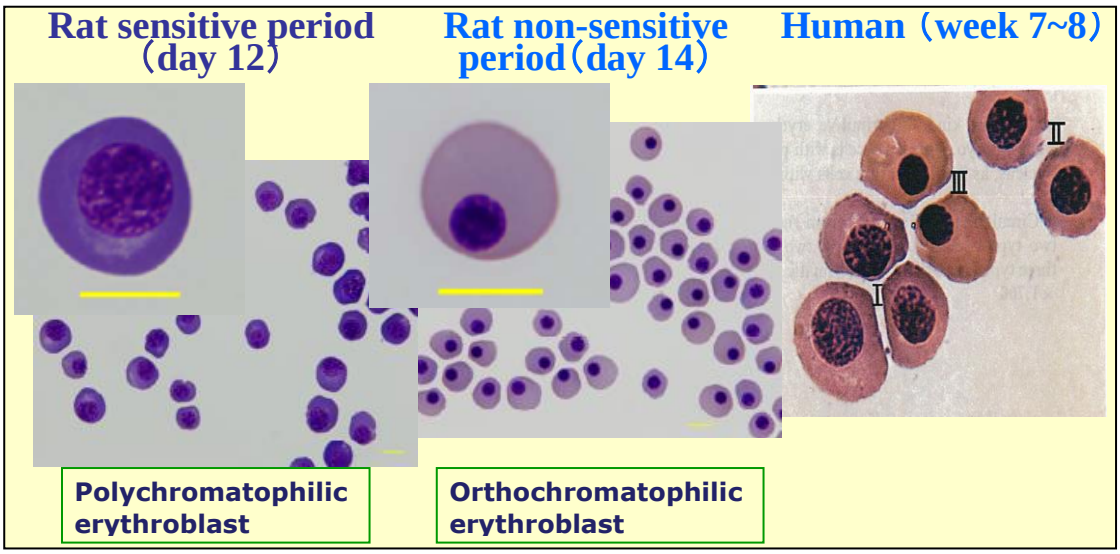


Fig. 4 Erythroblasts in the rat and human embryo

5.2 Vulnerability of erythrocytes exposed to various agents

It is known that rat erythrocytes are more fragile than human erythrocytes when exposed to osmotic imbalance, pH change (Matsuzawa 1979) and oxidative damage (McMillan 1995).

6. Variegate porphyria and artemisinins

Porphyrias are disorders in which the activities of the enzymes of the heme biosynthetic pathway, including PPO, are deficient. They can be classified as either hepatic or erythropoietic, depending on the principal site of expression of the specific enzymatic defect. The tissue-specific expression of porphyrias is largely due to the tissue-specific control of heme pathway gene expression, especially at the level of aminolevulinate synthase (ALAS), the first and rate-limiting enzyme of heme biosynthesis (Sassa 2000). In liver, hemoprotein enzymes are rapidly turned over in response to current metabolic needs. The activity of ALAS1, the housekeeping isoenzyme of ALAS, in normal liver is the lowest among all enzymes in the heme biosynthetic pathway. In erythroid cells, the activity of ALAS2 (the erythroid-specific isoenzyme of ALAS) is induced only during the period of active heme synthesis (Puy 2007).

Variegate porphyria (VP) is a disease associated with PPO deficiency. VP is categorized as hepatic porphyria. Hepatic porphyrias usually do not include anemia or hematological problems. This suggests that defective enzymatic activity resulting in disturbances in heme biosynthesis in liver does not necessarily limit heme synthesis in erythroid cells (Sassa 2000). According to Professor Meissner, whose laboratory has evaluated up to 3000 cases of VP patients over 35 years, there are no reports of the fetus presenting with symptoms of anemia, nor cardiac malformation.

It is therefore unlikely that PPO deficiency would induce anemia or disturbances of heme synthesis in human erythroid cells. . In contrast, the results of REL studies suggest that in rat erythroid cells, PPO activity is close to a rate-limiting enzyme activity. Therefore decreased PPO activity becomes rate-limiting in porphyrin production in erythroids resulting in PPIX accumulation, iron deposit, and anemia.

Artemisinins are anti-malaria drug and induce embryonic anemia resulting in developmental toxicity identical to flumioxazin. There is no species difference in developmental toxicity induced by artemisinins among rats, mice, rabbits, and monkeys. Artemisinins inhibit hemoglobin biosynthesis of human K562 cells by a mechanism unrelated to PPO inhibition (Fig. 5) (Finaurini 2012).In the latest paper by African researchers, 212 cases exposed to artemisinin during the first trimester are reported and the study has not shown any increase in either fetal death or malformations.

Flumioxazin,is completely different from artemisinins in terms of species difference. There is a clear species difference with flumioxazin whereas this is not the case with artemisinins. Human K562 cells are non-susceptible to flumioxazin unlike artemisinins. Thus even though the embryonic erythroblast is a target cell common to both flumioxazin and artemisinins, the mechanism of action is specific to the rat in the case of flumioxazin. It is therefore highly unlikely that humans are susceptible to the fetal anaemia and developmental effects of flumioxazin as seen in rats.

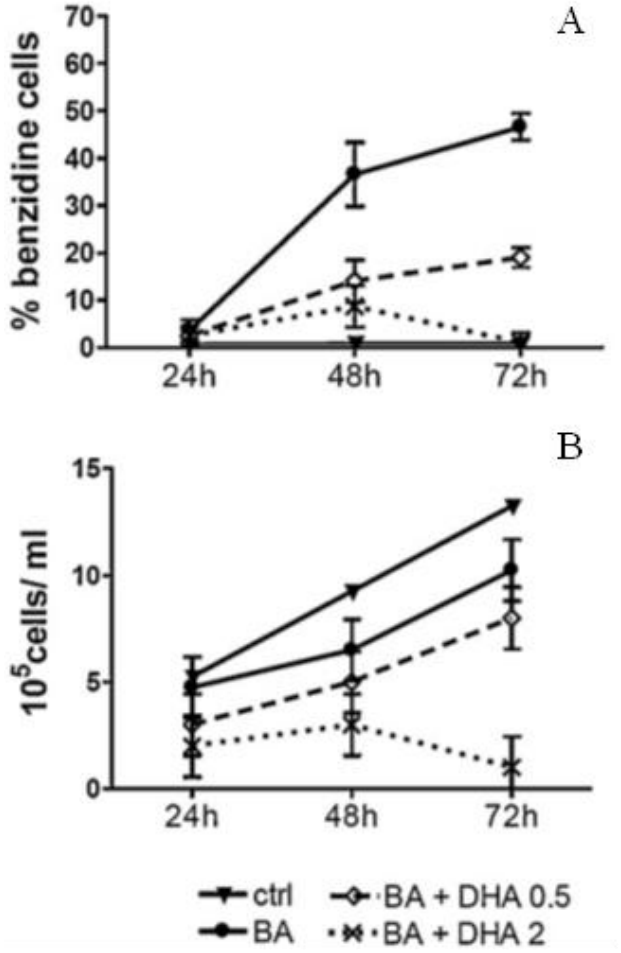


Fig. 5 Artemisinins inhibit haemoglobin biosynthesis of human K562
A to B indicate that time course of K562 induced erythroid differentiation (A) and cell proliferation (B) in the presence of dihydroartemisinin(DHA). Butyric acid (BA) was used for differentiation of K562 in A and B

7. Conclusions

The following conclusions are drawn based on the information presented above.

1. The *in vitro* rat REL erythroid cell is an appropriate model for assessing the effects on erythroids *in vivo*

2. Reduced heme biosynthesis is observed in REL cells at a concentration of 0.1 µM, close to that in embryos whose mother was exposed to 30 mg/kg flumioxazin and where PPIX accumulation is observed.

3. In contrast, in human fetal cells (CD36+ derived from cord blood) or human K562 cells, heme biosynthesis is not reduced at concentrations up to 5 µM where PPIX accumulates. The *in vitro* human cells are considered appropriate models for predicting the effects on erythroids *in vivo* in the human adult and fetus.

4. PPO activity could be close to that of the rate-limiting enzyme and reduced PPO activity becomes rate-limiting in rats, but not in humans. The overall difference in inhibitory activity of heme biosynthesis between rats and humans is greater than 50 times (0.1 µM vs 5µM).

5. The PBPK model is shown to be a justifiable approach to predict flumioxazin concentrations in the fetus of the pregnant human. The model shows that non susceptible concentrations of up to 5 µM in human erythroblastswould far exceed those attained in human embryos following flumioxazin exposure at a dose of 1000 mg/kg.

In conclusion, it is highly unlikely that humans are susceptible to the fetal anaemia and developmental effects of flumioxazin as seen in rats owing to the substantial qualitative and quantitative species differences between the rat and human.

8. References

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ANNEX 3
FLUMIOXAZIN AND VARIEGATE PORPHYRIA

Expert opinion from Dr Meissner

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6th February, 2014

TO WHOM IT MAY CONCERN

Flumioxazin and Variegate Porphyria

I have been asked to comment on the above issue by Odile Mercier, who to the best of my knowledge is a Toxicologist working for Sumitomo Chemical Agro Europe SAS in France.

I have never met Dr Mercier, and this view is expressly my own, the context and background of the issue has been explained in an email to me, asking for a scientific opinion. I have not been paid a consulting or any other fee, in cash or kind, for this comment.

I am a Medical Biological Scientist (PhD; Registered with the Health Professional Council of South Africa, registration #0002550). I have been asked to comment as I have spent my entire research career, since 1982, working on various aspects of the porphyrias, haem biosynthesis and the genes and enzymes associated with such.

I currently am the Head of Division of Medical Biochemistry at the University of Cape Town Medical School in South Africa, and Director of our specialist Porphyria Centre in the Dept. of Medicine.

I am not a clinician, but my area of work, means that I frequently work with clinicians, and am the leading expert in the diagnosis of the porphyrias in South Africa. I am frequentlyconsulted about both clinical treatment and biochemical aspects of porphyria. My CV is attached.

My lab is an affiliate member of the European Porphyria Network (EPNET) headed by Professor Sverre Sandberg at the University of Bergen in Norway. I have strong ties to the Porphyria Centre run by Profs Jean-Charles Deybach and Herve Puy in Paris, am a member of EPNET and have collaborated over many years with the UK Porphyria Centre, currently headed by Prof Michael Badminton in Cardiff, Wales. I consider myself a colleague of most porphyria experts around the world and have been on the organising committee of several international Porphyria congresses.

In my years I have interacted verbally or in person with several hundred variegate porphyria (VP) patients, and have diagnosed between 2- and 3000 in my lab. My lab currently diagnoses between 50 and 100 new cases of VP in South Africa. Many of these 'new cases' are in fact the children of VP families well-known to me through my work over the years. There is an historically high incidence of VP in South Africa due to a founder gene defect dating back to 1688. The research I am most well known for is the first description of this founder gene defect in protoporphyrinogen oxidase (PPO), a mutation commonly referred to as R59W.

VP is caused by an approximate 50% decrease in PPO activity, resulting from the inheritance of an inactive form of the protein, on one allele. The other allele is normal, thus PPO activity in VP patients is decreased, on average by 50%. It is an autosomal dominant condition.

ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON FLUMIOXAZIN (ISO); N-(7-FLUORO-3,4-DIHYDRO-3-OXO-4-PROP-2-YNYL-2H-1,4-BENZOXAZIN-6-YL)CYCLOHEX-1-ENE-1,2-DICARBOXIMIDE

To the best of our knowledge inheritance of an inactive form of PPO from both parents, resulting in 0% PPO activity is incompatible with life. Unpublished experiments from my lab. in which we cross-bred R59W heterozygous mutant PPO transgenic mice confirmed this. Approximately 25% of fetuses were in fact homozygous for R59W, none were born, and all were resorbed by day 12 of pregnancy and not born. The mechanism of fetal death/resorption was not studied.

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 VP patients show a loss of 50% of activity.

Considering the substrate for PPO is protoporphyrinogen-IX, there is primarily a build-up of protoporphyrinogen, which is assayed as the oxidised product, protoporphyrin. The porphyrinogen form is rapidly oxidised to the porphyrin form in the presence of oxygen and light. As protoporphyrin is the most fat-soluble of the various porphyrins, it is excreted mostly in the stool of patients. Other porphyrin intermediates also accumulate, such as coproporphyrin.

The correlation between excessive porphyrin production and clinical signs of the disease (skin photosensitivity and fragility in the sun-exposed parts of the body, face and back of hands) is not 100% pentrant. About 40% of patients present with clinical symptomology.

A much smaller (<5%) of patients may develop a potential fatal condition called the acute porphyric attack (an acute neuropathy) in response to excessive ALA and PBG excretion. During acute attack conditions all haem pathway intermediates are significantly over-produced and excreted, including the porphyrin precursors delta-aminolevulinic-acid (ALA) and porphobilinogen (PBG).

Various mechanisms of the acute attack have been proposed but the single common feature of all is that they link in some way to the presence of excessive ALA&PBG in these patient. The acute attack may be preciptated by drugs associated with porphyringenicity – those drugs resulting in an increase in activity of the rate-limiting enzyme of the pathway, ALA synthase. Thus, in addition to a VP patient having 50% deficiency in PPO, the haem pathway is further stimulated through induction of ALA synthase, which together with the primary inherited block in PPO results in a 'threshold' of flux through the pathway being breached, with resultant accumulation of porphyrin intermediates throughout the pathway, including their early precursors ALA and PBG.

By far the majority of patients with VP are able to live a relatively normal lifestyle, and their life-expectancy is normal. There are no reports of pregnancy-related problems in VP mothers related to porphyria. To my knowledge there are no reports of the fetus presenting with symptoms of anemia, nor cardiac malformation. Similarly children of VP patients, themselves carrying (or not) the VP gene do not present with specific symptoms of anemia any more so than in a normal population. I am unaware of any higher incidence of anemia in VP children nor in adulthood. We have some families in our database in whom we have monitored VP, from birth to their mid-forties, themselves having produced children. This comment is not based on formal study but because we have been a specialist porphyria centre, and the major referral centre in South Africa, since 1963. This routine follow up has been in place, in some cases for 45 years.

While all cells and tissue are carrying the defective gene for VP, it is primarily the liver where porphyrin over-production occurs as that is a tissue highly active in haem synthesis. Thus, in a sense all tissues suffer from the same abnormality but it is the case that VP (and the other acute porphyrias) are considered hepatic diseases. I have on numerous occasions measured PPO activity in lympoblastoid and fibroblasts derived from VP patients and the PPO defect

ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON FLUMIOXAZIN (ISO); N-(7-FLUORO-3,4-DIHYDRO-3-OXO-4-PROP-2-YNYL-2H-1,4-BENZOXAZIN-6-YL)CYCLOHEX-1-ENE-1,2-DICARBOXIMIDE

can be measured therein, as can deranged porphyrin metabolism be measured assuming one has sensitive enough fluorospectroscopic equipment.

I am unaware of VP in humans arising due to ingestion of PPO inhibitors, such as the diphenyl ether herbicides (e.g. acifluorfen) etc. Nevertheless, considering the fact that VP patients carrying an inherited PPO mutation exhibit anomalous porphyrin production, it would not be wise to expose such patients to further PPO (chemical) insult considering the possibility that further diminished PPO activity may on theoretical grounds give rise to severe porphyric symptomology. I do not think this has ever been tested or reported, but think that various PPO inhibitors are available in some markets (?) in the form of herbicides. I have no knowledge of differential sensitivity of human tissue, versus rat, mouse or any other cell types in response to PPO inhibitor treatment, nor differences in their uptake or clearance. That is not my field of expertise.

Various workers, including ourselves have used acifluorfen in the diet of mice to (a) either induce a chemical form of VP, or (b) to exacerbate porphyria symptoms in mice carrying the R59W gene defect. This is unpublished and unreplicated in other labs to me knowledge. PPO activity is negatively affected by such and excreted and hepatic porphyrin levels are increased. Depending on acifluorfen concentrations used the same, or higher levels of porphyrin excretion than in non-aciflurofen treated R59W mice can be achieved. We have been unable to induce any obviously observable acute neuropathic symptoms in such mice, with or without the PPO inhibitor. Thus while VP porphyrin biochemistry is inducible, acute symptomology is not under the conditions we used, nor can photosensitivity to any denuded areas on the mice observed. However, we have not systematically measured acute nor skin symptomology in these mice.

In summary, and in respect of the specific concerns surrounding possible fetal anemia and acrdiac malformation I can reiterate that I am unaware of suhc symptomolgies been reported in VP patients, or their children.

I have not had time to reference some of the above comments as perhaps they should be, and apologise for any typographical or grammatical errors in this statement. Time and my own priorities do not allow me to produce a perfect document.

I trust the above will suffice in answering the general and some fo the specific questions put to me by Dr Mercier in her original request of the 23rd January.

Sincerely

P.N. Meissner

Peter Meissner
Professor and Head: Division of Medical Biochemistry
Director: UCT Porphyria Labs, Dept. of Medicine

ANNEX 4
HUMAN RELEVANCY OF *IN VITRO* MODELS FOR ERYTHROPOIESIS

Embryo vs adult (primitive erythroid cells vs definitive erythroid cells)

There is a dramatic demand in haem synthesis during primitive erythropoiesis corresponding to the massive differentiation of erythroblasts in a synchronous manner. Fetuses are less sensitive to flumioxazin once passing this peak period since a major site of hematopoiesis shifts to the liver and continuing hematopoiesis starts. Adult daily red blood cell replacement is a few percent of whole volume. It is necessary that flumioxazin continues to affect erythropoiesis beyond compensable time period to produce anaemia in adults.

Haem biosynthesis would be common between primitive and definitive erythroids as haem is common between them although globin proteins are distinct. Inhibitory activity of flumioxazin on protoporphyrinogen oxidase (PPO) is comparable among embryonic on gestational day (GD) 12, fetal on GD 15, and adult liver PPOs (SBT-31-0045).

Primitive erythropoiesis shares many similarities with definitive erythropoiesis.

Hematopoietic development in vertebrates has been thought to comprise “primitive” and “definitive” phase. Primitive erythroid cells were thought to be “primitive” in that they seemed more closely resemble the nucleated erythroid cells of lower vertebrates than the enucleated erythrocytes of mammals, and were believed to be more “primitive” than adult red blood cells because they formed in the yolk sac, circulated as nucleated erythroblasts, and were present only transiently, during embryonic stages of development. It became evident that they are not so primitive. “Primitive” is a misnomer not only because embryonic, fetal and adult mammalian erythroid cells all enucleate but also because the lineages share a number of the other features, including differentiation from a unipotential progenitor, production of hemoglobin, progressive decrease in cell size and nuclear condensation during their terminal maturation, and regulation by erythroid transcription factors. They differ in some characteristics including the site of formation (primitive erythroid cells form only in the yolk sac) and expression of distinct globin genes (epsilon, zeta and gamma globins in human embryos; alpha and beta globins in adults) (1).

Primitive erythroblasts mature with progressive characteristics similar to their definitive counterparts, including 1) expansion of erythroblast numbers through a limited set of symmetric cell divisions, 2) accumulation of hemoglobin, 3) decrease in cell size, 4) nuclear pyknosis, and 5) decrease in RNA content (2).

Primitive and definitive erythroid cells can be partially separated by taking advantage of the difference in their size. However, there are presently no cell surface markers known that uniquely distinguish primitive from definitive erythroid cells. Primitive erythroid cells appear rapidly in the yolk sac as a large cohort of cells, maturing only after they enter the circulation. Red cells at all stages of development have many common features and, not surprisingly, they share expression of many of the same genes. Primitive and definitive erythroid cells differ in their requirements for certain transcription factors and may express distinct genes within multigene families such as those encoding globin, glucose transporters, and aquaporins (3).

Global gene expression was compared in primitive, fetal definitive, and adult definitive erythroid cells at morphologically equivalent stages of maturation purified from embryonic, fetal, and adult mice. Most genes active in erythroid cells are already expressed by the proerythroblast stage. Primitive and definitive erythroid lineages share a large set of genes (4).

Embryonic erythroblasts are not more highly sensitive to flumioxazin than adults.

K562 CD36+ vs primitive erythroid cells in human embryos

K562 cells are widely used to study proliferation and differentiation of hematopoietic cells.

Following induction of differentiation of K562 cells to erythroids, two major hemoglobin components were found in the cell lysate. Both were human embryonic hemoglobins (Hb). One was Hb-Portland (zeta2, gamma2) and the other was Hb-Gower I (zeta2, epsilon2). The electrophoretic pattern of K562 cell hemoglobin corresponds closely with that observed in hemoglobins from purified embryonic erythroblasts (5).

Another research group confirmed the observations that K562 cells accumulate human embryonic Hb after induction. In addition, it was demonstrated that K562 cells possess in their uninduced state fetal and embryonic globin chains, and globin mRNAs. K562 lacked several surface, enzymatic, and functional properties typical of granulocytes, lymphocytes, monocytes, or adult erythroblasts, including ABO and Rh blood groups, and adult Hb. 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. The phenotype expressed is more characteristics of early embryonic or fetal, as opposed to adult, hematopoietic cells (6).

K562 cells are sensitive to a chemical which causes anaemia in humans and a potential causative factor for anaemia.

Benzene exposure led to erythropoietic depression commonly observed in the human and animal individuals. It is widely accepted that the metabolites of benzene play important roles in the benzene toxicity to the hematopoietic system. Benzene metabolites decreased hemoglobin synthesis in K562 cells and inhibited the expression of transferrin receptor 1 and glycophorin A protein on the surface of K562 cells (7).

The iron chelator desferrioxamine inhibited haem accumulation and globin synthesis in K562 cells (8).

Primitive erythropoiesis shares many similarities with definitive erythropoiesis. A model of definitive erythropoiesis can serve as a useful model for the study of primitive erythroid maturation. Although primitive and definitive erythroid cells differ in the site of formation and expression of distinct globin genes, K562 cells produce embryonic globins and possesses primitive characteristics. In addition, K562 cells are sensitive to agents which cause anaemia in humans. Thus K562 cells are a good model to investigate effects on hematopoietic process in primitive erythroid maturation.

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