Bayer CropScience

REGULATORY TOXICOLOGY
POSITION PAPER

Subject :

FLUOPYRAM

CONTENTS :

Mode of Action and Human Relevance Analysis of Rodent Liver and Thyroid Tumors

Author:

[redacted]

Regulatory Toxicologist

[redacted]

Bayer CropScience

[redacted]

FRANCE

Date : 23/09/2013

INTRODUCTION

Fluopyram is a broad spectrum pyridylethylamide fungicide developed by Bayer CropScience (BCS) for global agricultural registration and use.

In the rat two year chronic/carcinogenicity study ([redacted], 2008) and mouse 18 month carcinogenicity study ([redacted], 2007), chronic exposure to fluopyram increased the incidence of liver adenomas and carcinomas in the female rat and thyroid follicular cell adenomas in the male mouse at the highest dose levels tested (Appendix; Table 1).

In July 2012, Germany, submitted the CLH report *Proposal for Harmonised Classification and Labelling* (version No.1) to the European Chemicals Agency (EChA) with the following proposal:

Risk phrase R40 (Carc. Cat. 3), "limited evidence of a carcinogenic effect" according to EU classification laid down in directive 67/548/EEC and hazard phrase H351 (Carc. 2) according to the new GHS-system (Regulation 1272/2008).

This proposal was based on the conclusion that uncertainties remained on the mode-of-action (MoA) data for rat liver tumors submitted by BCS that did not allow a conclusion to be drawn on the non-relevance of these tumors to humans. In contrast, the thyroid tumors in the mouse were deemed not to be of concern, as rodent thyroid tumors which are mediated by liver enzyme induction are well recognized as not posing a risk to humans and thus no classification was proposed.

Consequently, in order to address the concerns raised in the CLH report and to support registration of fluopyram globally, BCS has conducted a comprehensive mechanistic study programme that:

- Clearly demonstrates that fluopyram induces rodent liver and thyroid tumors via a non-genotoxic MoA that involves the activation of the constitutive androstane receptor (Car) and the pregnane X receptor (Pxr) in the liver.
- The MoA for fluopyram-induced rodent liver and thyroid tumors is comparable to phenobarbital, a well known Car-Pxr inducer.
- As for phenobarbital, the human relevance of the established MoA for the rodent tumors following exposure to fluopyram can be reasonably excluded based on fundamental qualitative differences in key events between experimental animals and humans, as shown by an *in-vitro* comparative study conducted on human and rat hepatocytes (Appendix; Figure 1).

- Other MoAs, such as involvement of AhR or PPARα receptors for the liver tumors, direct acting toxicants for the thyroid tumors and other potential contributors such as genotoxicity and cytotoxicity for both tumor types have been convincingly excluded.

This Position Paper presents a concise synopsis of the mechanistic data generated to elucidate the MoA for rat liver tumors and mouse thyroid tumors and the relevance of these tumors to humans. BCS believes that these data provide convincing evidence that fluopyram does not pose a risk as a potential human carcinogen and as such does not warrant an R40 (Carc. Cat. 3) / H351 (Carc.2) classification.

A MoA analysis, according to the 2006 International Programme on Chemical Safety (IPCS) for evaluating the weight of evidence for the carcinogenic MoA in animals followed by an analysis of the relevance of the cancer MoA observed in experimental animals for humans (Boobis *et al.*, 2006), is summarized in:
Table A*: Mode of Action Analysis for Rodent Tumors and Relevance to Humans,* on the following page.

An in depth review, including detailed information on the mechanistic studies conducted, is presented in the following Expert Summary Report: "Fluopyram: Mode of Action and Human Relevance Framework Analysis for Fluopyram-Induced Rodent Liver and Thyroid Tumors" ([redacted] *et al*, 2013).

Table A: Mode of Action Analysis for Rodent Tumors and Relevance to Humans

MoA	Liver tumors in rats	Thyroid tumors in mice
Postulated MoA	Car/Pxr nuclear receptor activation, measured by hepatic CYP enzyme induction (associated events; ↑ liver weight and hepatocellular hypertrophy), ↑ hepatocellular proliferation, ↑ altered hepatic foci → liver tumors	Car/Pxr nuclear receptor activation, measured by hepatic CYP enzyme induction, induction of specific UGT enzymes leading to an ↑ in T_4 clearance, resulting in HPT disturbance with a subsequent ↑ in TSH secretion, ↑ thyroid follicular cell proliferation, ↑ thyroid follicular cell hyperplasia → thyroid tumors
Key events	1. Activation of Car/ Pxr nuclear receptors and induction of hepatic CYP enzymes associated with liver hypertrophy 2. ↑ hepatocellular proliferation 3. ↑ altered hepatic foci, that eventually leads to liver tumors	1. Activation of Car/Pxr nuclear receptors and induction of hepatic CYP enzymes 2. ↑ specific UGT enzymes resulting in ↑ T_4 clearance 3. ↑ TSH 4. ↑ thyroid follicular cell proliferation 5. ↑ thyroid follicular cell hyperplasia, which eventually leads to thyroid tumors
Concordance of dose-response	Good correlation	Good correlation
Temporal association	Yes	Yes
Strength, consistency and specificity	Yes	Yes
Biological plausibility and coherence	Yes	Yes
Other MoA excluded	Genotox., cytotox., AhR or PPARα activation, ↑apoptosis, estrogens, statins, metals and infection mechanisms, all excluded	Genotox., cytotox., TPO inhibition, all excluded, induction of Car/Pxr nuclear receptors as demonstrated in WT versus Pxr-Car-KO mice study, indicate the involvement of an indirect MoA rather than a direct MoA.
Uncertainties, incon-sistencies and data gaps	No major inconsistencies and data gaps	No major inconsistencies and data gaps
Relevance to humans	Lack of proliferation in primary human hepatocytes exposed to fluopyram compared to rodent hepatocytes, indicates that the rat liver tumors associated with exposure to fluopyram are unlikely to occur in humans	WT versus Pxr-Car-KO mouse study provides compelling evidence for Car/Pxr being molecular initiating event for thyroid tumors. The rodent specificity of liver mediated thyroid toxicity due to differences in thyroid physiology between rodents and humans, indicates that the mouse thyroid tumors associated with fluopyram exposure are unlikely to occur in humans
Overall assessment and conclusion	MoA supported, liver tumors in rats not relevant to humans	MoA supported, thyroid tumors in mice not relevant to humans

OVERVIEW

Fluopyram Liver Tumor MoA

Based on the mechanistic and standard, repeat-dose toxicity studies in rodents the MoA for fluopyram-induced rodent liver tumors involves the activation of hepatic constitutive Car/Pxr nuclear receptors and is comparable to that of known Car/Pxr inducers such as phenobarbital. The relevant molecular and pathological endpoints for fluopyram-induced liver effects are summarized below and are consistent with the established key events of nuclear receptor-mediated rodent hepatocarcinogenesis (Lake, 2009; Cohen, 2010).

Liver Key Event #1

Key event #1 for the fluopyram-induced liver tumor MOA is defined as activation of Car/Pxr.

Activation of Car/Pxr is measured by surrogate liver-specific induction of the Car-specific Cyp2b and Pxr-specific Cyp3a cytochrome gene, protein, or correlative PROD, BROD/BQ enzymatic activity. Supportive, associative events to Key Event #1 include increased liver weight and microscopic hepatocellular hypertrophy. This key event occurs rapidly (significant changes are observed following 3 days of treatment) and has been well characterized in terms of temporal and dose response relationship (Appendix; Tables 2 & 3a). In addition, gene expression, enzyme activity, liver weight, and hepatocellular hypertrophy were reversible in rats after 28 days without exposure to the hepatocarcinogenic concentration of fluopyram.

One of the pivotal mechanistic studies conducted to demonstrate Car/Pxr receptor activation as the first key event was a 28-day mouse study ([redacted], 2013) using both the wild-type (WT) C57BL/6J mouse and a genetically modified mouse that does not have functional Car or Pxr receptors (hereafter referred to as Pxr-Car KO). Mice were exposed to fluopyram at the tumorigenic dose (750 ppm) in the mouse cancer bioassay and above (1500 ppm), equivalent in terms of mg/kg/d to the rat tumorigenic dose level. In this experiment, a significant induction of liver enzymes, liver enlargement and hepatocellular hypertrophy was seen in the WT mouse, but was not observed in the Pxr-Car KO mouse (Appendix; Table 4).

Liver Key Event #2

Key event #2 is defined as an increase in global hepatocellular proliferation.

This was seen in a temporal and dose-related manner in rats starting at 150 ppm, and was reversible to a significant degree at the top dose level of 1500 ppm, following a 28 day recovery period (Appendix; Table 5).

Liver Key Event #3

Key event #3 is defined as an increase in altered hepatic foci

Altered hepatic foci are considered to be proliferative pre-neoplastic lesions (Jones *et al*, 2009) that can over time develop into adenomas and carcinomas (Cohen, 2010). A significant increase in altered hepatic foci was recorded at the top dose (1500 ppm) of fluopyram at the end of both the chronic and carcinogenicity phase of the rat cancer bioassay.

All of the key events described for Car/Pxr-induced liver tumors were identified in fluopyram-exposed rats in a temporal- and dose-responsive manner with the final event of hepatocellular tumors observed in female rats exposed to 1500 ppm fluopyram. The earlier key events 1 and 2, were shown to be reversible on cessation of treatment.

Relevance of Rodent Liver Tumors to Humans

In order to demonstrate the non-relevance of rat liver tumors to humans, an *in-vitro* comparative study was conducted to examine the proliferative response in rat and human primary hepatocytes exposed to fluopyram at a range of dose levels up to a cytotoxic dose ([redacted] 2013[1] & 2013[2]). In a similar manner to phenobarbital, used as a positive control, the rat cells exposed to fluopyram showed a dose-response increase in proliferation, whereas human cells did not. Exposure to epidermal growth factor, a positive control, resulted in a marked proliferative response in both rat and human cells (Appendix; Figure 1).

This *in-vitro* experiment clearly demonstrates that one of the crucial key events for progression of liver tumors (key event 2; hepatocellular proliferation) would not take place in humans.

Other Modes of Action

Other plausible MoAs for liver tumor formation that are likely to be relevant to humans (Cohen, 2010) have effectively been excluded (Appendix; Table 6).

DNA reactivity is the first broad category of a MoA for hepatocellular carcinogens. A battery of *in-vitro* genotoxicity studies; the bacterial reverse mutation test (Ames test), mammalian chromosome aberration test, and a mammalian cell gene mutation test, together with an *in-vivo* mouse micronucleus assay showed that fluopyram does not have a genotoxic potential. Thus, DNA reactivity can be excluded as a potential MoA for the induction of tumors in rats or mice.

For non-DNA-reactive rodent liver carcinogens, several MoAs have been identified that act by stimulating hepatocellular proliferation through either a receptor- or non-receptor-mediated mechanism. The MoA studies in rats and mice with fluopyram clearly demonstrate a specific, dose-related increase in the Cyp2b/Car-associated and Cyp3a/Pxr-associated gene and enzyme activity. Furthermore, the absence of such a response in Pxr-Car-KO mice supported the specificity for fluopyram-induced activation of Car and Pxr and excluded other receptor (Pparα, AhR, ER, statins, and cytotoxicity) and non-receptor-mediated (cytotoxicity, infections, iron overload, and increased apoptosis) involvement in the key events leading to fluopyram-induced liver tumors. The increase observed in Cyp1a1 gene transcript levels, a non-specific indicator of AhR activation, led to a mimimal increase in EROD enzymatic activity (Appendix; Tables 3a & 3b), indicating that activation of this receptor is most unlikely to be a key driver in promoting fluopyram induced liver tumors in the rat.

Fluopyram Thyroid Tumor MoA

The MoA for fluopyram-induced rodent thyroid effects is similar to that for known Car/Pxr inducers. The use of Pxr-Car-KO mice has demonstrated that activation of Car and Pxr in the liver is obligatory to induce thyroid alterations following exposure to fluopyram (Appendix; Table 5). The relevant molecular and pathological endpoints for fluopyram-induced thyroid effects are summarized below and are coherent with the established key events of Car/Pxr-mediated rodent thyroid tumorigenesis (Dellarco *et al.*, 2006; Hurley *et al.*, 1998).

Thyroid Key Event #1

Key event #1 for fluopyram-induced thyroid tumor MoA is defined as activation of Car/Pxr.

Activation of Car/Pxr was observed by increases in the Phase I enzymes PROD (specific for Car) and BQ (specific for Pxr) at ≥ 30 ppm following fluopyram exposure for 28 days. On cessation of treatment for 28 days at the tumorigenic dose level of 750 ppm, enzyme activities returned to control levels. As expected in Pxr-Car-KO mice, no relevant changes in PROD and BQ were observed.

Thyroid Key Event #2

Key event #2 is defined as UGT induction leading to increased T_4 clearance and decreased plasma T_4.

In mice, the induction of the UGT-associated enzymes was not seen until ≥ 150 ppm following fluopyram exposure for 28 days. This shows that a threshold of exposure between 75 and 150 ppm fluopyram in mice is required before the liver requires the detoxification services provided by the UGT-associated enzymes. On cessation of treatment for 28 days at the tumorigenic dose level of 750 ppm, enzyme levels returned to normal.

Additionally, Pxr-Car-KO mice showed no induction of UGT-associated Phase II enzymes after exposure to a tumorigenic concentration of fluopyram for 28 days. Indications of increased T_4 clearance were observed in mice pre-exposed to fluopyram at 2000 ppm for up to 4 days prior to injection with radio-labelled T_4. Further evidence for effects on T_4 levels was provided by several studies in which decreases in plasma T_4 were observed.

Thyroid Key Event #3

Key event #3 is an increased level of TSH.

A significant induction of *Tsh b* in the pituitary gland of mice was observed at ≥ 600 ppm following fluopyram exposure for 28 days. Following cessation of treatment for 28 days at the tumorigenic dose level of 750 ppm, the levels of this transcript returned to normal. When *Tsh b* was examined in Pxr-Car-KO mice exposed to a carcinogenic concentration of fluopyram for 28 days, no change in transcript levels was seen, consistent with the lack of change in levels of UGT-associated enzymes.

Thyroid Key Event #4

Key event #4 is an increase in thyroid follicular cell proliferation.

A dose response increase in follicular cell proliferation was observed in mice exposed to fluopyram for 28 days starting from 150 ppm. Following cessation of treatment for 28 days at a dose level of 1500 ppm, which was higher than the tumorigenic dose of 750 ppm, follicular cell proliferation rates returned to normal. However, Pxr-Car-KO mice exposed to the tumorigenic concentration of fluopyram for 28 days showed no increase in follicular cell proliferation, thus demonstrating that activation of these receptors is required for altered thyroid effects.

Thyroid Key Event #5

Key event #5 is an increase incidence of thyroid follicular cell hyperplasia seen after chronic administration of fluopyram to mice.

This effect was observed at ≥ 150 ppm, following treatment for 12 months or more.

Overall the key events for Car/Pxr-induced thyroid tumors were identified in fluopyram-exposed mice in a temporal and dose-responsive manner, with the final event of thyroid adenomas observed in male mice exposed to 750 ppm fluopyram after a period of 18 months. Compelling evidence for Car/Pxr being the molecular initiating event for these tumors is provided by the absence of the key events in Pxr-Car-KO mice when exposed to fluopyram. The earlier key events 1 to 4, were shown to be reversible on cessation of treatment.

Relevance of Rodent Thyroid Tumors to Humans

Rodent thyroid tumors which are secondary to induction of liver enzymes are widely accepted as being non-relevant for humans due to differences in thyroid physiology between rodents and humans (Capen, 1997; Dellarco et al 2006;). The main reasons for the difference in response between rodents and humans are as follows:

I. Rodents are more sensitive to thyroid hormone changes
II. Rodents have enhanced thyroid hormone elimination
III. Thyroxine binding globulin is major plasma protein in humans (which acts as a buffer), but not in rodents
IV. Consequence, the concentration of unbound T_4 is greater in rodents than humans, resulting in greater susceptibility to metabolism and excretion and compensatory increase in thyroid follicular cell turnover, which over time can result in thyroid tumors.

Other Modes of Action

Other plausible MoAs for thyroid tumor formation have effectively been excluded (Appendix; Table 7).

The induction of chemical-specific thyroid tumors in rodents may be caused by the following MoAs (Capen, 1997; Hurley *et al.*, 1998):

I. DNA reactivity
II. Inhibition of the active transport of inorganic iodide into the follicular cell (iodide pump)
III. Inhibition of thyroid peroxidase that converts inorganic iodide into organic iodide and couples iodinated tyrosyl moieties into thyroid hormone
IV. Damage to follicular cells
V. Inhibition of thyroid hormone release into the blood
VI. Inhibition of the conversion of T_4 to T_3 by 5'-monodeiodinase at various sites in the body
VII. Enhancement of the metabolism and excretion of thyroid hormone by the liver, largely through the action of UGT

Concerning MoA I; *DNA reactivity*, this mechanism can be ruled out based on *in-vitro* and *in-vivo* genotoxicity studies which show that fluopyram is not a genotoxic chemical. MoA II - V are the result of direct thyroid gland effects, whereas VI and VII are peripheral to the thyroid gland. A direct MoA is unlikely based on the pivotal Pxr-Car-KO study which provides compelling evidence to demonstrate that fluopyram is a Car/Pxr inducer and is not a direct thyroid toxicant. More specifically, concerning MoA III; *Inhibition of thyroid peroxidase*, mechanistic studies using hog thyroid microsomes showed fluopyram did not affect thyroid peroxidase ([redacted], 2008). With regard to MoA IV; *Damage to follicular cells*, microscopic examination of the thyroid gland in the mouse and rat have never shown overt cytotoxicity, only hyperplasia/adenoma in the mouse following chronic treatment and hypertrophy and hyperplasia in the rat. As for the indirect MoA VI; *Inhibition of the conversion of T_4 to T_3 by 5'-monodeiodinase at various sites in the body*, is unlikely, as serum levels of T_3 were not changed in either the mouse or rat exposed to fluopyram. This indicates that the indirect MoA VII; *Enhancement of the metabolism and excretion of thyroid hormone by the liver, largely through induction of UGT enzymes*, is the most likely mechanism for thyroid tumor induction and is strongly supported by the mechanistic data.

CONCLUSION

Overall, the mechanistic studies together with the standard repeat dose studies, clearly demonstrate the MoA for the rodent liver and thyroid tumors following a life-time exposure to fluopyram is *via* activation of the Car/Pxr nuclear receptors. Furthermore, the lack of proliferation in primary human hepatocytes exposed to fluopyram as compared to rodent hepatocytes, coupled with the rodent specificity of liver mediated thyroid toxicity, provide convincing evidence that both the liver and thyroid rodent tumors are non-relevant to humans. Therefore, applying a weight of evidence analysis, it can be concluded that fluopyram does not pose a risk as a potential human carcinogen and as such does not warrant an R40 (Carc. Cat. 3) / H351 (Carc.2) classification.

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APPENDIX

Table 1: Liver and thyroid tumors from carcinogenicity studies in rat and mice exposed to fluopyram

	Wistar rat							
	Male				Female			
Dose (ppm) [mg/kg bwt/day]	**0 -**	**30 [1.20]**	**150 [6.0]**	**750[a] [29]**	**0 -**	**30 [1.68]**	**150 [8.6]**	**1500 [89]**
# Examined	60	60	60	58	59	57	56	55
# with ≥ 1 liver adenoma	2	1	2	1	2	2	0	**9**
# with ≥ 1 liver carcinoma	0	0	0	0	0	0	2	3
# with ≥ 1 liver adenoma/carcinoma(s)	2	1	2	1	2	2	2	**11[b]**
	C57BL/6J mice							
	Male				Female			
Dose (ppm) [mg/kg bwt/day]	**0 -**	**30 [4.2]**	**150 [20.9]**	**750 [105]**	**0 -**	**30 [5.3]**	**150 [26.8]**	**750 [129]**
# Examined	49	47	48	48	48	50	50	50
# with ≥ 1 thyroid adenoma	1	1	3	**7**	3	1	3	1
# with ≥ 1 thyroid carcinoma	0	0	0	0	0	0	0	0

Bold represents statistically significant differences from the relevant controls.[a] : Dose reduced to 375 ppm from week 85 onwards; [b] : One animal had both adenoma and carcinoma.

APPENDIX

Table 2: Gene expression given as mean fold change relative to controls for female Wistar rats exposed to fluopyram for 3, 7, or 28 days with a recovery group of 28 days.

	Dose (ppm)	30	75	150	600	1500
Associated receptor	Rat Genes	**3 days**				
Ahr	*Cyp1a1*	-1.2	1.1	1.7	**7.3**	**62.7**
Car	*Cyp2b1*	-1.6	1.1	**3.3**	**49.6**	**244.1**
Pxr	*Cyp3a3*	1.1	**1.5**	**2.6**	**8.2**	**21.5**
Pparα	*Cyp4a1*	-1.1	NC	-1.1	NC	-1.3
Associated receptor	Rat Genes	**7 days**				
Ahr	*Cyp1a1*	1.4	1.8	**4.6**	**63.6**	**222.9**
Car	*Cyp2b1*	2.6	3.1	**14.4**	**326.5**	**1434.0**
Pxr	*Cyp3a3*	1.5	**1.9**	**3.6**	**12.4**	**28.6**
Pparα	*Cyp4a1*	-2.1	-2.4	-2.3	-2.3	**-3.2**
Associated receptor	Rat Genes	**28 days**				
Ahr	*Cyp1a1*	1.8	2.3	**8.1**	**100.9**	**354.7**
Car	*Cyp2b1*	2.7	1.7	**10.9**	**212.5**	**1543.8**
Pxr	*Cyp3a3*	**1.8**	**3.7**	**5.3**	**17.1**	**50.4**
Pparα	*Cyp4a1*	-1.2	-1.1	NC	-1.2	-1.4

Dose (ppm)	28 day 1500	28 day Recovery 1500	% Change[a]
Cyp1a1	**354.7**	1.8	↓100%
Cyp2b1	**1543.8**	1.7	↓100%
Cyp3a3	**50.4**	**2.6**	↓95%
Cyp4a1	-1.4	1.1	NC

NC = no change. Bold represents statistically significant differences from the relevant controls. Three and 7 day data are from BCS report SA 10240 and the 28 day and 28 day recovery data are from BCS report SA 11104, [redacted] 2011 ; [redacted] 2012, respectively. a: Formula for % change: [1- (recovery treated-recovery control)/(treated – control)]*100 using values of mean relative quantity as given in report SA 11104. Alternate direction of sign in fold change value between treated and recovery group reported as 100% change.

APPENDIX

Table 3a: Enzyme activity given as fold change in female Wistar rats exposed to fluopyram for 7 or 28 days with a recovery period of 28 days at 1500 ppm

Mean fold change relative to controls			
Fluopyram dose (ppm)	**EROD**	**PROD**	**BROD**
	7 days		
30	1.1	1.3	1.1
75	0.9	1.5	1.2
150	1.0	1.4	1.4
600	1.1	**2.2**	**2.4**
1500	**1.6**	**4.6**	**5.9**
	28 days		
30	1.1	0.9	1.2
75	1.1	1.3	1.4
150	**1.3**	1.5	2.9
600	**1.3**	**1.9**	**9.1**
1500	**2.0**[a]	**4.8**	**39.1**

Rat 28 day Recovery	**Mean Fold change Relative to Controls.**		
Fluopyram dose	**EROD**	**PROD**	**BROD**
1500 ppm	**1.2**	**1.5**	**1.5**
% Change[a]	↓78%	↓86%	↓99%

Bold represents statistically significant differences from the relevant controls. Data from BCS reports SA 10240 (7 days) and SA 11104 (28 days), [redacted] 2011 ; [redacted] 2012, respectively a: % Change compared to 1500 ppm fluopyram at the end of 28 day exposure. Formula for % change: [1- (recovery treated -1)/(treated -1)]*100 using values of mean relative quantity as given in report SA 11104.

[a] Maximum elevation of EROD was 2-fold, which is considered to be a minimal increase. In BCS studies examining EROD levels in rats exposed to the known AhR agonist β-naphthoflavone, EROD levels were increased 9.1-fold, data are presented in Table 4 on the following page.

APPENDIX

Table 3b: EROD enzyme activity in male and female Wistar rats exposed to dietary fluopyram for 28 days. Additionally, EROD levels for the positive control β-Naphtoflavone (β-NF) exposed via oral gavage at 75 mg/kg/day for 28 days.

Rat 28 day	Males				Females			
Fluopyram dose (ppm) [mg/kg/d]	**50 [4]**	**400 [31]**	**3200 [254]**	**β-NF [75]**	**50 [4.6]**	**400 [36.1]**	**3200 [263]**	**β-NF [75]**
EROD fold-change	-1.3	1.1	1.3	**7.2**	1.0	1.3	1.7	**9.1**

Rat fluopyram data is from BCS study SA 03332, [redacted], 2004. β-Naphtoflavone data is from BCS study SA 06181, [redacted], 2007.

Bold represents statistically significant differences from the relevant controls.

APPENDIX

Table 4: Comparison of fluopyram induced changes in WT and Pxr-Car KO male mice

		750 ppm fluopyram/28 days dietary	
Corresponding Key Event	Parameter	WT	Pxr-Car-KO
1 (Liver & thyroid tumors)	Hepatic Phase I enzyme activity	↑ PROD/BQ	No change
2 (Thyroid tumors)	Hepatic Phase II enzyme activity	↑UGT-T4/UGT-BIL	No change
3 (Thyroid tumors)	Tsh β	↑	No change
4 (Thyroid tumors)	Thyroid follicular cell proliferation	↑	No change

APPENDIX

Table 5: Temporal / dose response for total hepatocellular proliferation in female Wistar rats given as % change from control.

Fluopyram Dose ppm (Study No.)	3 Days (SA 10240)	7 Days (SA 10240)	28 Days (SA 11104)
30	-13	41	-11
75	12	19	18
150	**86**	**106**	**70**
600	**267**	**192**	**67**
1500	**551**	**257**	**188**
Bold indicates statistically different from relevant controls.			

Rat 28 day Recovery Phase (SA 11104)	**Total hepatocellular proliferation**
Fluopyram dose	
1500 ppm % change from control	51

Data from BCS reports SA 10240 (7 days) and SA 11104 (28 days),

[1]▇▇▇▇▇ 2011 ; [2]▇▇▇▇▇ 2012, respectively

APPENDIX

Table 6: Summary Evaluation for Other Possible MoAs for liver tumor formation

	DNA Reactivity	AhR or Pparα Activation	Cytotoxicity (3-90 Day Rat studies)	Increased Apoptosis	Estrogens, Statins, Metals, Infectious
Strength of Association	All standard battery of genotoxicity tests were negative	Increase in Cyp1a1 gene expression, non-specific indicator of AhR activation, led to a minimal increase in EROD, therefore unlikely to be key event leading to fluopyram induced liver tumors No increase in Cyp4a1gene expression, an indicator of Pparα activation.	No changes in relevant clinical chemistry parameters & no hepatic focal necrosis	No histopath evidence; however difficult to determine microscopically	No histopath evidence, structural dissimilarity to estrogens
Consistency of Association	-	-	-	-	-
Specificity of Association	-	-	-	-	-
Dose-Response Concordance	No tumors at lower doses in rats	-	-	-	-
Temporal Relationship	Late onset tumors	-	-	-	-
Coherence & Plausibility	Not Plausible; Not Coherent	Not Plausible; Not Coherent	Not Plausible; Not Coherent	Not Plausible; Not Coherent	Not Plausible; Not Coherent

- indicates attribute absent

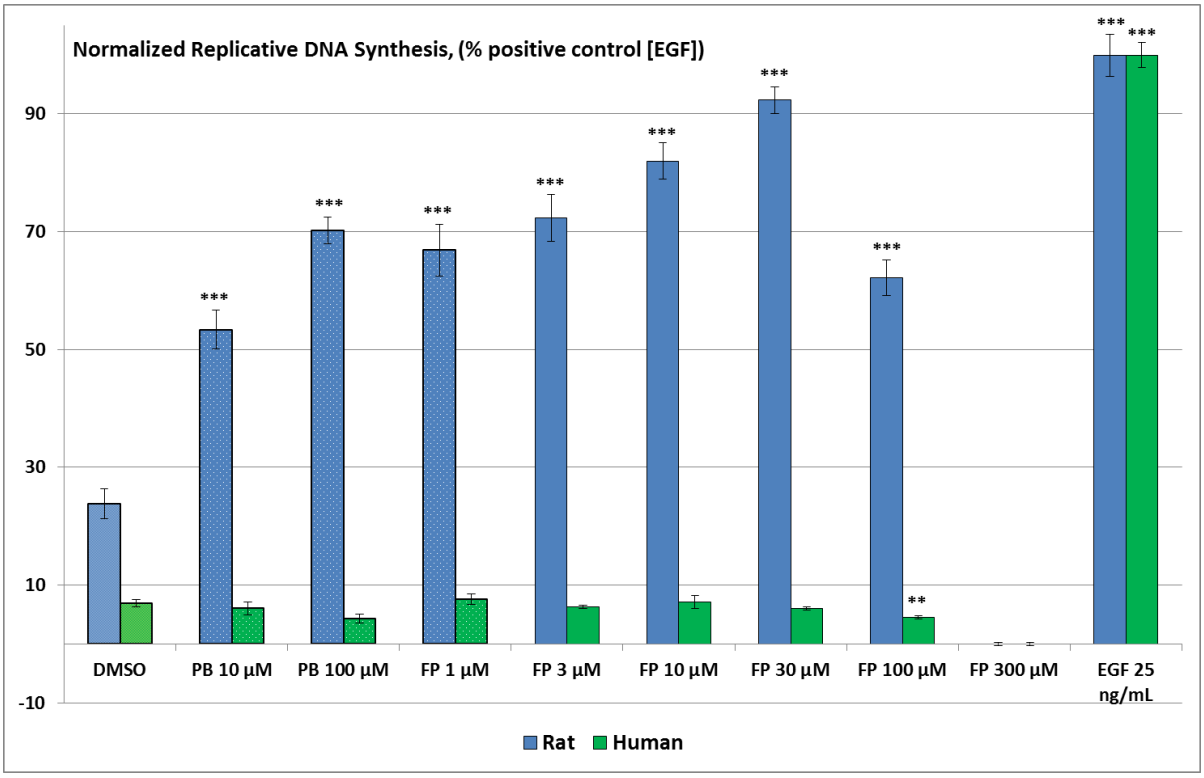
APPENDIX

Table 7: Summary Evaluation for Other Possible MoAs for Thyroid tumor formation

	DNA Reactivity	Damage to Follicular cells	Inhibition of thyroid peroxidase	Other Direct MoA	Inhibiton of T_4 to T_3 via indirect MoA
Strength of Association	All genotoxicity tests were negative	No histopath evidence of overt cytotoxicity in thyroid in rodent studies	Mechanistic studies using hog thyroid microsomes showed fluopyram did not affect thyroid peroxidase	Unlikely based on mechanistic data strongly indicating indirect MoA via Cxr-Pxr activation	Unlikely as serum levels of T_3 unchanged in rodent studies
Consistency of Association	-	-	-	-	-
Specificity of Association	-	-	-	-	-
Dose-Response Concordance	No tumors at lower doses in mice	-	-	-	-
Temporal Relationship	Late onset tumors	-	-	-	-
Coherence & Plausibility	Not Plausible; Not Coherent	Not Plausible; Not Coherent	Not Plausible; Not Coherent	Not Plausible; Not Coherent	Not Plausible; Not Coherent

APPENDIX

Figure 1: DNA-Synthesis Induction in Human and Rat Hepatocytes



Proliferative response in rat and human primary hepatocytes exposed to fluopyram (FP) given as normalized replicative DNA synthesis compared to epidermal growth factor (EGF). Phenobarbital (PB) and EGF were used as positive controls. Fluopyram was slightly cytotoxic at 100 µM with 100% mortality at 300 µM

$p<0.05$; ** $p<0.01$; *** $p<0.001$.