Study Title

Fluopyram:
Mode of Action and Human Relevance Framework Analysis for Fluopyram-Induced Rodent Liver and Thyroid Tumors

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Signature Page

Compound: Fluopyram
Title: Fluopyram: Mode of Action and Human Relevance Framework Analysis for Fluopyram-Induced Rodent Liver and Thyroid Tumors

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Table of Contents

Statement of No Data Confidentiality Claims 2
Compliance with Good Laboratory Practice Standards 3
Signature Page 4
Table of Contents 5
Summary 7
1. Introduction 9
2. Key Events: Dose-response and Temporal Association 16
Liver Tumor MOA and Key Events 16
Key Event #1: Car/Pxr receptor activation with induction of hepatic cytochrome P450 gene expression and enzyme activity 16
Key Event #2: Increased hepatocellular proliferation 20
Key Event #3: Altered hepatic foci 21
Liver Associative Events 23
Mouse Supportive Data 25
Summary of Fluopyram Liver Tumor MOA 28
Thyroid Tumor MOA and Key Events 30
Key Event #1: Liver Car/Pxr activation with induction of Phase I metabolic enzymes 32
Key Event #2: Phase II liver enzyme induction leading to increased serum T_4 clearance and consequently decreased circulating T_4 34
Key Event #3: Increased TSH 37
Key Event #4: Increased thyroid cell proliferation 39
Key Event #5: Increased thyroid cell hyperplasia 41
Mouse Thyroid Associative Events 41
Rat Supportive Data 43
Summary of Fluopyram Thyroid Tumor MOA 45
3. Strength, Consistency, and Specificity of Association of Tumor Response with Key Events 48
4. Biological Plausibility and Coherence. 50
5. Other MOAs. 53
6. Uncertainties, Inconsistencies, and Data Gaps. 65

7. Assessment of Postulated Mode of Action.....70

8. Applying the International Programme on Chemical Safety Human Relevance Framework to Fluopyram. 70

Question 1. Is the weight of evidence sufficient to establish the MOA in animals? **.....70**

Question 2. Can human relevance of the MOA be reasonably excluded based on fundamental qualitative differences in key events between experimental animals and humans?.....71

Question 3. Can human relevance of the MOA be reasonably excluded based on quantitative differences in either kinetic or dynamic factors between experimental animals and humans?**.....75**

Conclusions.....76

References.....78

Appendix (in order by earliest year) 86

Summary

In carcinogenicity studies, fluopyram caused liver tumors in the female rat and thyroid tumors in the male mouse. The proposed mode-of-action (MOA) involves activation of the constitutive androstane receptor (Car) and the pregnane X receptor (Pxr). The MOA for the liver tumors includes the following key events: 1) activation of the Car/Pxr receptors and increased activity of hepatic cytochrome P450 (CYP) enzymes, 2) increased hepatocellular proliferation, and 3) increased incidence of altered hepatic foci that progress to liver tumors. The MOA for the thyroid tumors includes the following key events: 1) activation of the Car/Pxr receptors and increased activity of hepatic cytochrome P450 enzymes, 2) increased activity of specific uridine glucoronyltransferases (UGTs) associated with thyroxine (T_4) thyroid hormone clearance, 3) increased levels of thyroid stimulating hormone (TSH), 4) increased follicular cell proliferation and 5) increased thyroid follicular cell hyperplasia that eventually proceeds to thyroid tumors. These key events have been evaluated in a series of MOA studies aimed at providing data to perform a weight-of-evidence evaluation using the Bradford-Hill criteria with subsequent application into a Human Relevance Framework (HRF). The conclusion from this evaluation is that fluopyram-induced rodent liver and thyroid tumors are via a non-genotoxic MOA that involves the activation of hepatic Car and Pxr. Activation of these nuclear receptors produced a cascade of alterations in gene transcription that led to increased activity of hepatic CYP and UGT enzymes and increased hepatocellular proliferation, the latter being a critical event in the development of liver tumors. Subsequently, the increased hepatic enzyme activity, particularly of the UGTs, seen in fluopyram exposed mice following activation of Car/Pxr led to decreased levels of thyroid hormones, primarily T_4. This decrease in thyroid hormones resulted in increased TSH secretion from the pituitary, which led to increased follicular cell proliferation, hyperplasia and eventually adenomas in the thyroid. The MOA responsible for the tumors seen in fluopyram exposed rodents is similar to that for other Car/Pxr inducers. Evidence for CAR/PXR induction being the common molecular initiating event for the two tumor types is compelling as studies have shown that fluopyram exposed Pxr-Car knockout mice were refractory to the early liver changes as well as to the TSH changes and thyroid follicular cell proliferative effects.

Considering the dose response relationships for each of the key events, there is convincing evidence that the MOA for fluopyram-induced thyroid and liver effects in the mouse and rat, respectively, do not occur below a threshold dose level. Specifically, the two key events of the MOA prior to tumor formation in the rat liver, increased cell proliferation and altered hepatic foci, occurred at ≥ 150 ppm, with tumors at 1500 ppm. In the mouse, the two key events of the MOA prior to tumor formation in the thyroid, increased thyroid follicular cell proliferation and hyperplasia, started from 150 ppm, with tumors at 750 ppm. Consequently fluopyram should be considered as a threshold carcinogen and on this basis, a threshold approach rather than a quantitative (Q*) assessment for risk assessment should be applied to the mouse thyroid and rat liver tumors associated with fluopyram exposure.

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 indicate that the rat liver and mouse thyroid tumors associated with fluopyram exposure is unlikely to occur in humans.

1. Introduction

Common name: Fluopyram

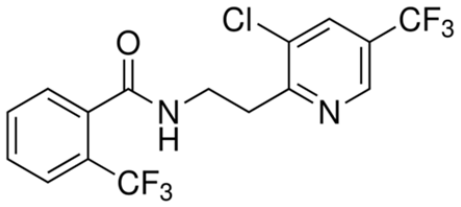
International Union of Pure and Applied Chemistry [IUPAC]) name: (N-(2-[3-chloro-5-(trifluoromethyl)-2-pyridinyl]ethyl)-2-(trifluoromethyl)benzamide)

Chemical Abstracts Service [CAS] Number: 658066-35-4

Molecular formula: $C_{16}H_{11}ClF_6N_2O$

Molecular weight: 396.72 g/mol

Structure:



Fluopyram is a broad spectrum pyridylethylamide fungicide intended for global agricultural registration and use. Fluopyram controls fungi by inhibiting the enzyme succinate dehydrogenase, which is a functional part of the tricarboxylic acid cycle linked to mitochondrial electron transport.

Registration of new plant pesticides, *e.g.,* herbicides, insecticides, fungicides, requires comprehensive mammalian toxicity assessment to evaluate acute oral/dermal toxicity, skin and eye irritation, skin sensitization, genetic toxicity, systemic or target organ toxicity, developmental and reproductive toxicity, neurotoxicity, and carcinogenesis. Requirements for the conduct of these studies differ slightly among regulatory agencies globally, but in principle, a comprehensive evaluation of mammalian toxicity is required to identify potential hazards to human health as well as to derive the reference doses used for human risk assessment. One of the cornerstone studies used for pesticide registration is the two-species rodent cancer bioassay. The carcinogenic hazard, if any, identified in these studies along with the no observed-adverse-effect levels (NOAELs) are often used to drive the overall human health risk assessment and derive the reference doses.

Chronic exposure to fluopyram increased the incidence of liver adenomas and carcinomas in the female rat and increased the incidence of thyroid follicular cell adenomas in the male mouse (Table 1).

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

	Wistar rat							
	Male				Female			
Dose (ppm)	0	30	150	750[a]	0	30	150	1500
# 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)	0	30	150	750	0	30	150	750
# 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
# with ≥ 1 thyroid adenoma/carcinoma(s)	1	1	3	**7**	3	1	3	1

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 .

In the initial cancer assessment of fluopyram by the U.S. Environmental Protection Agency (US EPA), the Cancer Assessment Review Committee (CARC) stated: "…that insufficient data were provided to support definitive modes of action for the induction of liver tumors in female rats or thyroid follicular cell tumors in male mice. The main deficiency included a lack of dose-response concordance with key events and tumors" (US EPA, 2009). Canada's Pest Management Regulatory Agency (PMRA) made a similar conclusion. As a consequence, the US EPA has allocated a Q* to fluopyram based on the rat liver tumors, while PMRA has allocated a Q* based on the mouse thyroid tumors. Furthermore, the CARC classified fluopyram as "Likely to be Carcinogenic to Humans" based on tumors in two species and two sexes: a treatment-related increase in thyroid follicular cell adenomas in high-dose male mice and liver

tumors in high-dose female rats, with incidences exceeding that of the laboratory's historical controls.

Most chemicals that cause rodent liver tumors can be classified according to their specific mode of action (MOA), and a rational evaluation can be made in terms of the relevance to humans based on this information (Cohen, 2010). Several MOAs have been identified for liver carcinogenesis in both rodents and humans, with those applicable to the latter given in bold in Table 2.

Table 2. Modes of Action for Hepatocellular Carcinogenesis (Cohen, 2010).

I. DNA reactivity
II. Increased cell proliferation
  a. Receptor mediated
    i. Ppar (peroxisome proliferation)
    ii. Enzyme induction (Car, Pxr, AhR)
    iii. Estrogen
    iv. Statins
    v. Cytotoxicity
    vi. Other
  b. Non-receptor mediated
    i. Cytotoxicity
    ii. Infectious
    iii. Iron (copper) overload
    iv. Increased apoptosis (e.g., fumonisin B1)
    v. Other

Modes of action in **bold** letters are likely to be relevant to humans.

From the battery of genetic toxicology studies, it is known that fluopyram is not mutagenic or genotoxic. 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. Evidence from early studies suggested that the profile of liver effects induced by fluopyram were similar to those induced by phenobarbital, a molecule known to exert its effects through activation of the constitutive androstane receptor (Car) and the pregnane X receptor (Pxr) in the liver. Compounds that induce both Car/Pxr can also disrupt thyroid hormone balance, leading

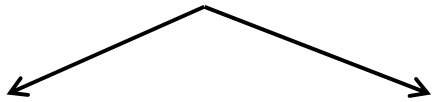
to stimulation of the thyroid cells, which can eventually result in formation of thyroid tumors following chronic exposure (Hiasa *et al.*, 1982; McClain *et al.*, 1988).

Postulated MOA for liver and thyroid tumor formation

The recommended requirements for assessment of the Car/Pxr activation MOA (Cohen, 2010; [redacted] 2013; Dellarco *et al.*, 2006) are described in detail below.

Key Events for Car/Pxr-Mediated Liver and Thyroid MOA.

(1) Car/Pxr receptor activation in liver
Altered hepatic gene expression
Induction of hepatic Phase I and II enzymes



LIVER TUMORS	THYROID TUMORS
(2) Increased hepatocellular proliferation. (3) Increase in altered hepatic foci.	(2) Increased T_4 clearance. (3) Increase TSH. (4) Increased thyroid follicular cell proliferation.

Key events 1 and 2 for the liver and 1 to 4 for the thyroid are all reversible on discontinuance of treatment.

Mechanistic/MOA studies were conducted to demonstrate these key events following exposure to fluopyram as well as to characterize the dose and temporal -response relationships for each of the key events. As liver tumors occurred only in the female rat and thyroid tumors only in the male mouse, the mechanistic studies described in this document focused primarily on female rat liver and male mouse thyroid to reduce the number of animals utilized to understand the MOA of fluopyram. As the induction and cascade of Car/Pxr response is similar in mice and rats, gleaning a mechanistic understanding by reading across both gender and species is scientifically justified. In addition to the mechanistic studies, results from repeat-dose rat and mouse studies with fluopyram are supportive of the postulated MOA.

In this document, the data obtained from all of these studies are presented according to the 2006 International Programme on Chemical Safety framework 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).

Listing of repeat dose and liver/thyroid specific mechanistic MOA studies: Please see the appendix for summaries.

[redacted] (2004); AE C656948 - Exploratory 28-day toxicity study in the rat by dietary administration; Bayer S.A.S., Bayer CropScience; Report No.: SA 03332; Document No.: M-085510-01.

[redacted] (2004); AE C656948 - Preliminary 28-day toxicity study in the mouse by dietary administration; Bayer S.A.S., Bayer CropScience; Report No.: SA 04013; Document No.: M-088486-01.

[redacted] (2005); AE C656948 – 90-day toxicity study in the rat by dietary administration; Bayer S.A.S., Bayer CropScience; Report No.: SA 04048; Document No.: M-250946-01.

[redacted] (2005); AE C656948 – 90-day toxicity study in the mouse by dietary administration; Bayer S.A.S., Bayer CropScience; Report No.: SA 04052; Document No.: M-251136-01.

[redacted] (2007); Carcinogenicity study of AE C656948 in the C57BL/6J mouse by dietary administration; Bayer S.A.S., Bayer CropScience; Report No.: SA 05094; Document No.: M-295688-01.

[redacted] (2008); Chronic toxicity and carcinogenicity study of AE C656948 in the Wistar rat by dietary administration; Bayer S.A.S., Bayer CropScience; Report No.: SA 04312; Document No.: M-298339-01.

[redacted] (2008); Fluopyram (AE C656948) - 7-day mechanistic study in the female Wistar rat by dietary administration; Bayer S.A.S., Bayer CropScience; Report No.: SA 07323; Document No.: M-299274-01.

[redacted] (2008); Phenobarbital - 7-day mechanistic study in the female Wistar rat by gavage; Bayer S.A.S., Bayer CropScience; Report No.: SA 07325; Document No.: M-299491-01.

[redacted] (2008); AE C656948 (Fluopyram) - *In vitro* studies on the potential interactions with thyroid peroxidase-catalyzed reactions; Bayer HealthCare AG; Report No.: AT04481; Document No.: M-299276-01.

[redacted] (2008); AE C656948 - Mechanistic 14-day toxicity study in the mouse by dietary administration (hepatotoxicity and thyroid hormone investigations); Bayer S.A.S., Bayer CropScience; Report No.: SA 07215; Document No.: M-299522-01.

[redacted] (2008); Phenobarbital - Mechanistic 14-day toxicity study in the mouse by oral gavage (hepatotoxicity and thyroid hormone investigations); Bayer S.A.S., Bayer CropScience; Report No.: SA 07326; Document No.: M-299521-01.

[redacted]. (2008); AE C656948 - Mechanistic 3-day toxicity study in the male mouse (pharmacokinetic investigations of the clearance of intravenously administered 125I-thyroxine); Bayer S.A.S., Bayer CropScience; Report No.: SA 08159; Document No.: M-308369-01.

[redacted] (2008); AE C656948 - Mechanistic 3-day toxicity study in the male mouse (qPCR investigations of gene transcripts in the liver); Bayer S.A.S., Bayer CropScience; Report No.: SA 08151; Document No.: M-308073-01.

[redacted] (2009); AE C656948 - Definitive mechanistic 4-day toxicity study in the male mouse (pharmacokinetic investigations of the clearance of intravenously administered 125I-thyroxine); Bayer S.A.S., Bayer CropScience; Report No.: SA 08288; Document No.: M-328662-01.

[redacted] (2011); Fluopyram – Mechanistic 3-day toxicity study in the mouse by oral gavage (thyroid hormone investigations); Bayer S.A.S., Bayer CropScience; Report No.: SA 10241; Document No.: M-408352-01.

[redacted] (2011); Fluopyram - Mechanistic investigations in the female rats by dietary administration for up to 7 days; Bayer S.A.S., Bayer CropScience; Report No.: SA 10240; Document No.: M-408029-01.

[redacted] (2012); Fluopyram – Mechanistic 3-day toxicity study in the mouse by oral gavage (thyroid hormone investigations); Bayer S.A.S., Bayer CropScience; Report No.: SA 10430; Document No.: M-426994-01.

[redacted] (2012); Fluopyram – Mechanistic 28-day toxicity study in the mouse by dietary administration (hepatotoxicity and thyroid hormone investigations); Bayer S.A.S., Bayer CropScience; Report No.: SA 11105; Document No.: M-428031-02.

[redacted] (2012); Fluopyram - Mechanistic investigations in the liver of female rats following dietary administration; Bayer S.A.S., Bayer CropScience; Report No.: SA 11104; Document No.: M-427431-01.

[redacted] (2012); Fluopyram – 28-day toxicity study for proliferation assessment in the C57BL/6J male mouse; Bayer S.A.S., Bayer CropScience; Report No.: SA 11123; Document No.: M-428303-01.

[redacted] (2013). Fluopyram: 28-day toxicity study for thyroid cell proliferation in the C57BL/6J male mouse Bayer S.A.S., Bayer CropScience; Report Number: SA 12058; Document No.: M-449821-03.

[redacted] (2013). 28-Day dietary study to determine potential role of the nuclear preganane X receptor (Pxr) and constitutive androstane receptor (Car) on the thyroid changes following the administration of fluopyram to male mice (C57BL/6J and Pxr KO/Car KO); Bayer S.A.S., Bayer CropScience; Report Number: SA 12162; Document No.: M-449890-01.

[redacted] (2013). Fluopyram: Comparative assessment of enzyme and DNA synthesis induction in cultured rat hepatocytes; Report Number: CXR1242; Document No.: M-450157-01

[redacted] (2013). Fluopyram: Comparative assessment of enzyme and DNA synthesis induction in cultured human hepatocytes; Report Number: CXR1241; Document No.: M-450156-01.

[redacted] (2013). Fluopyram: Assessment of pentoxyresorufin-*o*-depentylation and benzyloxyquinoline-*o*-debenzylation in 50 liver microsomal samples; Report Number: CXR1284; Document No.: M-451628-01.

2. Key Events: Dose-response and Temporal Association

Liver Tumor MOA and Key Events

Key Event #1: Car/Pxr receptor activation with induction of hepatic cytochrome P450 gene expression and enzyme activity

The first key event is activation of Car/Pxr nuclear receptors and evidence for this first key event is generally provided by increased expression of specific hepatic cytochrome P450 genes and activation of their associated enzyme products. In particular, activation of Car/Pxr is associated with the induction of the Cyp2b (Car) and Cyp3a (Pxr) families (Ueda *et al.*, 2002) and their corresponding enzymes as measured by pentoxyresorufin-O-depentylation (PROD) and benzyloxyresorufin-O-debenzylation (BROD) or benzyloxyquinoline (BQ), respectively. For example, the prototypical Car/Pxr inducer phenobarbital shows marked increases in these parameters in rodents following exposure.

Associative evidence that can aid in substantiation of Car and Pxr activation (and hence a Car/Pxr-mediated MOA) includes Phase II enzyme induction (eg glucuronosyltransferases and glutathione S-transferases) and liver weight increases accompanied with microscopic hepatocellular hypertrophy, which is commonly seen following exposure to phenobarbital-like compounds. These changes are reversible following discontinuance of treatment.

During the early evaluation of fluopyram, increased PROD and BROD were observed in both sexes from 400 ppm in a 28-day rat study indicating activation of Car and Pxr, respectively (Table 3). A similar profile was also observed in a preliminary mechanistic study where female rats were exposed to 3000 ppm fluopyram for 7 days.

Table 3. Enzyme activity shown as fold change compared to control for male and female Wistar rats exposed to fluopyram for either 28 days (n = 5 per group) or 7 days (females only; n = 10 per group).

	28 days						7 days
	Males			Females			Females
Fluopyram dose (ppm)	50	400	3200	50	400	3200	3000
EROD	-1.3	1.1	1.3	1.0	1.3	1.7	**2.2**
PROD	-1.4	*4.5*	*10.4*	1.2	*4.6*	*16.3*	**4.3**
BROD	1.1	*2.3*	*19.3*	1.5	*9.4*	*31.0*	**11.7**

Bold represents statistically significant differences from the relevant controls. Italics represent data considered as biologically significant (no statistical analyses conducted). Data are from BCS reports SA 03332 (28 days) and SA 07323 (7 days; females only).

Further evidence for Car/Pxr activation is provided from two additional mechanistic studies where quantitative polymerase chain reaction (qPCR) and enzyme activity were used to characterize the dose and temporal response of Phase I (AhR-, Car-, Pxr-, or Pparα-associated) transcripts/enzymes in female rats following treatment with fluopyram. In the first study, the animals were exposed to fluopyram in the diet for either 3 or 7 days. In the second study, exposure was for 28 days with additional control and top dose group females being maintained on control diet for a further 28 days after the exposure period. The dietary levels used in these studies covered the range of levels tested in female rats in the chronic cancer bioassay, *i.e.,* 30, 150, and 1500 ppm. Summaries of the gene expression data and the enzyme activity data are presented in Tables 4 and 5, respectively.

Gene Expression

The Phase I gene expression changes show a dose-response increase in Car/Pxr-related genes (*Cyp2b1* and *Cyp3a3*) due to fluopyram treatment and show a common profile observed for other Car/Pxr inducers (Ueda *et al.*, 2002; Juberg *et al.*, 2006; Kodama and Negishi, 2006). Additionally, elevated transcript levels of *Cyp1a1* were seen with fluopyram, suggesting possible direct activation of the AhR or activation through

cross-talk with the more significantly induced Car/Pxr. *Cyp4a1* was not elevated in these studies, highlighting the lack of Pparα induction.

A return to normal transcript levels for Phase I enzyme-related genes was recorded for animals exposed to fluopyram for 28 days at the tumorigenic dose and then placed on a control diet for a further 28 days. Overall, the results of these gene expression studies demonstrated dose and temporal responses (3–28 days) of Car/Pxr.

Table 4. 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. 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.

Enzyme Activity

Rats exposed to fluopyram showed a temporal and dose-related induction of Car/Pxr-related enzyme activities that corresponded with the previously presented gene expression data. PROD and BROD were significantly elevated at doses ≥ 600 ppm in the female rat (Table 5). The slight increase in liver 7-ethoxyresorufin-O-deethylase (EROD) activity corresponds to the slight induction of *Cyp1a1* gene expression. All of these findings are consistent with earlier studies (SA 03332; SA 07323).

A 1500 ppm exposure group that was allowed to recover for 28 days showed a significant reduction in these enzyme activities (78–100%).

Table 5. 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**	**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). 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.

CONCLUSIONS FOR KEY EVENT 1 (Car/Pxr receptor activation with induction of hepatic cytochrome P450 gene expression and enzyme activity):

- Key Event 1 of the liver tumor MOA has been identified.
- Dose responses have been established for Car (Cyp2b1) and Pxr (Cyp3a3) gene expression and enzyme activity.
- Car/Pxr associated enzymes (PROD and BROD) were increased at ≥400 ppm (SA 03332). In the mechanistic studies (SA 10240; SA 11104) statistically significant changes in Car/Pxr associated gene transcripts were observed from 30 ppm.
- The changes associated with Key Event 1 are reversible following cessation of fluopyram.
- These findings support activation of the Car and Pxr nuclear receptors.

Key Event #2: Increased hepatocellular proliferation

Typically, Car/Pxr inducers such as phenobarbital increase hepatocellular proliferation within 2 to 3 days of treatment then slowly return to "normal" levels of proliferation after about 4 - 6 weeks (slightly longer for mice) (Kolaja *et al.*, 1996a; Yamada *et al.*, 2009).

In a preliminary study, female rats were exposed to 3000 ppm fluopyram for 7 days, and a significant increase in liver proliferation (increased 4x)[BCS study SA 07323] was recorded (measured by BrdU incorporation). Additional temporal- and dose-response evaluations of hepatocellular proliferation in female rats were performed by Ki-67 immunohistochemical staining of labeled nuclei as a measure of hepatocellular proliferation (Table 6).

The greater degree of proliferation at 3 days versus 7 days shows the typical early wave of proliferation that peaks at around 3 days for fluopyram as for other known Car/Pxr inducers. Proliferation is slower between 3 and 7 days because the liver has already reached an enlarged (hepatomegaly) state and is adapting to adequately meet metabolic/detoxification demand. These findings are supported by the histopathological observation of increased mitosis in around 25% of the high dose females at 3 days, whereas none was seen at 7 days. The threshold for significant induction of hepatocellular proliferation at 3, 7, or 28 days was ≥ 150 ppm. The significant

proliferation recorded at ≥ 150 ppm after 28 days of exposure shows fluopyram affects proliferation in a sustained manner.

Table 6. Liver Key Event #2: Temporal / dose response for total hepatocellular proliferation in female Wistar rats given as % change from control.

Temporal →

Dose ↓

Dose ppm	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.

The recovery data for animals placed on the control diet for 28 days following a 28-day exposure at 1500 ppm demonstrate that the proliferative effects induced by fluopyram were reversible. In the 1500 ppm group, total hepatocellular proliferation was 51% higher than control at the end of the recovery period and was approximately 50% lower than the amount of proliferation observed after 28 days of continuous treatment.

CONCLUSIONS FOR KEY EVENT 2 (Increased hepatocellular proliferation):

- Key Event 2 for liver tumor MOA has been identified.
- Dose responses have been established and a significant increase in hepatocellular proliferation is observed at ≥ 150 ppm.
- The changes associated with Key Event 2 are reversible following discontinuance of fluopyram.

Key Event #3: Altered hepatic foci

The chronic administration of Car/Pxr inducers, such as phenobarbital, result in the development of altered hepatic foci (IARC, 2001; Jones *et al.*, 2009; Thorpe and Walker, 1973; Whysner *et al.*, 1996). These focal lesions of hepatocytes are characterized by altered cytoplasmic tinctorial properties that can be classified as either basophilic,

eosinophilic, clear cell, or mixed type (reviewed in [redacted] *et al.*, 1994). Altered hepatic foci are classified as proliferative, preneoplastic lesions that can result from sustained hepatocellular proliferation. They are encountered in control animals and appear progressively during the lifetime of the rodents. In studies using F344/NCr rats (Ward, 1983), and C3H/He, C57BL/6 (Evans *et al.*, 1986, 1992), and B6C3F1 (Kolaja *et al.*, 1996c) mice, the liver lesions produced by phenobarbital were predominantly eosinophilic in nature. This observation is also seen with fluopyram.

Most rodent liver carcinogens increase the size and/or number of altered hepatic foci prior to the appearance of tumors and are believed to be precursors of liver tumors (Popp and Goldsworthy, 1989). Phenobarbital administration results in a dose-dependent increase in cell proliferation within foci that is associated with progression from altered hepatic foci to hepatocellular adenomas (Klaunig, 1993). Altered hepatic foci (Table 7) at the conclusion of the carcinogenicity studies for fluopyram were significantly greater than controls both in the female and the male rat (81 and 48 % occurrence at the top dose, respectively). In the rat chronic study (12 months), the incidence of altered hepatic foci was higher in the male compared to the female (50 and 30%, respectively). However, after 21 months on study, the high dose of 750 ppm demonstrated adverse clinical signs and mortality in the male rat required lowering of this dose to 375 ppm to ensure sufficient numbers of surviving males at the scheduled sacrifice to allow appropriate statistical analyses. This resulted in equivalent altered hepatic foci in males at 24 months (50 versus 48%), whereas an increase from 30 to 81% was recorded for the female rat (maintained at 1500 ppm fluopyram) at 12 and 24 months, respectively. If the male rats could have continued the carcinogenicity study at 750 ppm, it is highly probable that they would have developed liver tumors as was seen in the female rat.

Although development of altered hepatic foci is listed as a key event for Car/Pxr induced hepatic tumors, these pre-neoplastic growths are a reflection of the sustained proliferative and metabolic demand which is the primary biological response leading to selective clonal expansion, formation of altered hepatic foci, and the subsequent development of adenomas and/or carcinomas.

Table 7. Altered (eosinophilic) hepatic foci from Wistar rats exposed to fluopyram for 12 or 24 months.

Rat		Males				Females			
12 months	Dose (ppm)	0	30	150	750	0	30	150	1500
	# Examined	10	10	10	10	10	10	10	10
	% Altered hepatic foci	20	10	20	*50*	0	0	0	*30*
24 months	Dose (ppm)	0	30	150	375	0	30	150	1500
	# Examined	60	60	60	58	60	60	60	59
	% Altered hepatic foci	27	40	**52**	**48**	48	43	50	**81**

Data is from BCS report SA 04312. Bold represents statistically different compared to relevant controls. Italics considered biologically significant in the absence of statistics.

CONCLUSIONS FOR KEY EVENT 3 (Altered hepatic foci):

- Key Event 3 for liver tumor MOA has been identified.
- A threshold of response at 1500 ppm has been identified in female rats for the occurrence of altered hepatic foci.

Liver Associative Events

Additional associative events that inform the degree of Car/Pxr-mediated hepatic effects are liver weight and hypertrophy data. Increased liver weight and hepatocellular hypertrophy are commonly observed in rodents exposed to Car/Pxr inducers (Juberg *et al.*, 2006).

Rat Associative Events—Female rats exposed to fluopyram for 3, 7, or 28 days showed significantly increased liver weight from 150 ppm (Table 8). Following 90 days treatment liver weight continued to be increased from 200 ppm; however following chronic treatment (12 or 24 months) statistically significant increases were only recorded in the high dose (1500 ppm) groups. Hypertrophy was observed in all studies starting from 400 ppm (SA 03332) (Table 9). These effects (weight increase and hypertrophy) were completely reversible in rats treated at 1500 ppm fluopyram for 28 days followed by a 28-day recovery period on a control diet. In addition, hepatocellular hypertrophy was found to be reversible for rats exposed to 3200 ppm fluopyram for 90 days followed by a 28 day recovery period.

Table 8. Temporal / dose response with recovery for increased relative liver weights in **female Wistar rats** given as % increase over controls.

Temporal →

Dose ↓

Dose ppm	**3 days** (SA10240)	**7 days** (SA10240)	**28 days** (SA11104; SA 03332)	**90 days** (SA04048)	**28 day Recovery** (SA11104; SA 04048)	**12 months** (SA04312)	**24 months** (SA04312)
30	0%	-5%	2%			1%	-3%
50			0%	0%			
75	2%	-3%	5%				
150	4%	-2%	**7%**			4%	6%
200				**11%**			
400			15%[A]				
600	**5%**	3%	**13%**				
1000				**27%**			
1500	**17%**	**18%**	**33%**		3%	**54%**	**56%**
3200			**73%**	**74%**	**13%**		

Blank cell = No data. **Bold** indicates treatment-related and statistically significant increases in liver weight A: was considered treatment-related but was not statistically significantly different from controls

Table 9. Temporal / dose response for hepatocellular hypertrophy in **female Wistar rats** given as % incidence.

Temporal →

Dose ↓

Dose ppm	**3 days** (SA 10240)	**7 days** (SA 10240)	**28 days** (SA 11104; SA 03332)	**90 days** (SA 04048)	**28 day Recovery** (SA 11104; SA 04048)	**12 months** (SA 04312)	**24 months** (SA 04312)
30	0	0	0			0	0
50			0	0			
75	0	0	0				
150	0	0	0			0	0
200				0			
400			**20**				
600	0	7	**40**				
1000				**70**			
1500	**40**	**93**	**93**		0	**100**	**81**
3200			**100**	**100**	0		

Blank cell = no data. **Bold** indicates treatment-related and biologically significant in absence of statistical analyses

Mouse Supportive Data

Although the tumor and primary mechanistic data that provide information on the MOA for liver tumors were derived from the rat model, additional information can be gleaned from studies conducted in the mouse.

Male mice exposed to 2000 ppm fluopyram showed a significant increase in Car/Pxr-related PROD/BROD enzyme activity at 3 and 14 days (Table 10).

Table 10. Enzyme activity shown as fold change compared to control for male C57BL/6J mice exposed to fluopyram for 3 or 14 days.

Mouse	3 days	14 days
Fluopyram dose (ppm)	2000	2000
PROD	**29.1**	**22.6**
BROD	**88.2**	**91.6**

N= 5 samples per group where each sample represents a pool of the livers of 3 animals. Data is from BCS report SA 07215. Bold represents statistically significant differences from the relevant controls.

Mice exposed to fluopyram in a 28 day dose response study showed a significant increase in PROD and BQ enzyme activity from the lowest dose tested (30 ppm) (Table 15).

Data from wild-type (WT) and Pxr-Car knockout (KO) mice exposed to a tumorigenic concentration of fluopyram (750 ppm) revealed a dichotomous enzyme response (Table 16). WT mice had elevated PROD and BQ enzyme activities. Pxr-Car-KO mice also showed a slight but significant induction in PROD (1.4-fold); however, this was 50 times lower than the activity seen in the WT mouse (69.8-fold). BQ levels in the Pxr/Car-KO mice were mildly but significantly reduced (-1.5-fold) compared to the induction seen in the WT animals (5.5-fold). Importantly, the Pxr/Car-KO mouse was constructed using the same background strain (C57BL/6J) that was used in the fluopyram mouse carcinogenicity study.

Mouse Associative Events

As with the rat, increased liver weight and hepatocellular hypertrophy associated with Car/Pxr activation have been observed in the mouse. The temporal and dose response for the associative events of increased liver weight and hepatocellular hypertrophy in male mice are summarized in Tables 11 and 12, respectively.

Increased liver weight was first observed in a standard 28 day study from 150 ppm and in a mechanistic 28 day study, a significant increase in liver weight was observed ≥ 75 ppm (Table 11). Mice exposed to fluopyram for 90 days and longer (12 and 18 months) continued to show increased liver weight.

An increase in the incidence of hepatocellular hypertrophy was seen starting from 150 ppm in male mice (Table 12). In a study with WT and Pxr-Car-KO mice exposed to 750 ppm fluopyram for 28 days, a significant increase in liver weight was observed in the WT, whereas only a slight but significant increase was seen in KO mice (Table 13). In addition, hepatocellular hypertrophy was observed in WT but not in the KO mice.

Although fluopyram caused thyroid but not liver tumors in the mouse, all of the key events for Car/Pxr-induced liver tumorigenesis were seen at the thyroid tumorigenic concentration of 750 ppm. These data demonstrate consistency with the liver key events seen in rodents following exposure to known Car/Pxr inducers.

Table 11. Temporal / dose response for increased relative liver weights in male C57BL/6J mice given as % increase over controls.

Temporal →

Dose ↓

Dose ppm	**3 days** (SA 07215)	**14 days** (SA 07215)	**28 days** (SA 11105; SA 04013)	**90 days** (SA 04052)	**12 months** (SA 05094)	**18 months** (SA 05094)
30			4%	0%	0	6
75			**6%**			
150			**9% - 21%**[A]	14%	**15%**	**14%**
600			**27%**			
750 (Recovery)			**33%** (4%)		**25%**	**31%**
1000			**46%**	**36%**		
2000	**61%**	**61%**				

Blank cell = No data. Bold indicates treatment-related, statistically significant increase in liver weight. A: common dose between SA 11105 and SA 04013

Table 12. Temporal / dose response for hepatocellular hypertrophy in male C57BL/6J mice given as % occurrence.

Temporal →

Dose ↓

Dose ppm	3 days (SA 07215)	14 days (SA 07215)	28 days (SA 04013)[A]	90 days (SA 04052)	12 months (SA 05094)	18 months (SA 05094)
30				0	0	0
150			**100%**	**100%**	**63%**	**78%**
750			**100%**		**100%**	**100%**
1000				**100%**		
2000	**100%**	**100%**				

Blank cell = No data. **Bold** indicates treatment-related and biologically significant in absence of statistical analyses. A: Liver histopathological analyses were not performed in the 28d study SA 11105

Table 13. Mean liver to body weight (bw) ratio given as % change, enlarged liver and hepatocellular hypertrophy reported as % occurrence for male wildtype and Pxr-Car knockout (KO) mice exposed to fluopyram at 750 ppm for 28 days.

Mouse	Wildtype		Pxr-Car KO	
ppm	0	750	0	750
Mean liver to bw ratio (% change)	0	**39**	0	**7**
Enlarged liver (% occurrence)	0	*47*	0	0
Hepatocellular hypertrophy (% occurrence)	0	*100*	0	0

Data from BCS report SA 12162. Bold represents statistically significant differences from the relevant controls. Italics considered biologically significant in the absence of statistics.

Summary of Fluopyram Liver Tumor MOA

Based on the mechanistic and standard, repeat-dose toxicity studies in rats treated with fluopyram the MOA for fluopyram-induced rodent liver tumors is comparable to that of known Car/Pxr inducers (eg 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, which 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 dose response relationship. 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. Evidence of this key event is compelling as Pxr-Car KO mice showed almost no induction of Phase I enzyme activity and no evidence of liver enlargement, or hepatocellular hypertrophy after 28 days exposure to fluopyram at a dose level that was equivalent in terms of mg/kg/d to the rat tumorigenic dose level.

Liver Key Event #2

Key event #2 is an increase in global hepatocellular proliferation that was seen in a dose-related manner in rats starting at 150 ppm, which was reversible following a 28 day recovery period (73% of the levels seen at 28 days). The induction of liver proliferation is the crucial key event in the progression of tumor formation and the differing response between rodents and higher mammals. This finding is also consistent with the known MOA for Car/Pxr-mediated rodent liver tumorigenesis.

Liver Key Event #3

Key event #3 is an increase in altered hepatic foci that are considered to be proliferative pre-neoplastic lesions (Jones *et al.*, 2009). Persistent hepatocellular proliferation leads to the induction of these lesions within the liver that can over time develop into adenomas and carcinomas (Cohen, 2010). A significant increase in altered hepatic foci was recorded at the top dose of fluopyram at the end of both the chronic and carcinogenicity phase of the 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.

Thyroid Tumor MOA and Key Events

A consequence of increased liver metabolism in rodents exposed to Car/Pxr inducers is increased elimination of thyroid hormones, which are produced by the thyroid and monitored by the hypothalamus-pituitary-thyroid axis. The hypothalamus releases thyrotropin-releasing hormone (TRH), which stimulates TSH release. The anterior pituitary gland secretes thyroid-stimulating hormone (TSH), which induces the production and release of thyroxine (T_4) and triiodothyronine (T_3) by the thyroid gland. The expression of TSH and TRH is controlled through a negative-feedback process that is very sensitive to circulating T_4 and T_3 levels. When the liver is exposed to high doses of a Car/Pxr inducer, it responds by increasing the size and number of cells as well as the activity of those enzymes capable of detoxifying the compound. Some of these detoxification enzymes, in particular the Phase 2 enzymes uridine diphosphate glucuronyltransferases (UGTs) and sulfotransferases, conjugate the Car/Pxr inducer with respectively a glucoronide or a sulfate moiety. This conjugation allows an easier elimination of the compound *via* urinary and biliary clearance (Klaassen and Hood, 2001). A consequence of this increased UGT and sulfotransferase activity is not only the increased elimination of xenobiotics but also the increased conjugation and subsequent elimination of T_4, resulting in decreased serum thyroid hormone levels. As part of a feed-back mechanism increased TSH is secreted by the pituitary gland to stimulate the thyroid gland to increase production of thyroid hormones to restore homeostasis. A sustained increase in thyroid hormone production is often achieved through hypertrophy and proliferation of thyroid follicular cells. After chronic exposure to high doses of a Car/Pxr inducer, hyperplasia and eventually tumors in the thyroid gland may occur due to sustained over-stimulation by TSH (Hiasa *et al.*, 1982; McClain *et al.*, 1988).

Compounds that induce both Car and Pxr in rodents are known to result in decreased circulating levels of thyroid hormones, which following lifetime exposures can result in thyroid tumors (Hiasa *et al.*, 1982; McClain *et al*., 1988). The effects on the thyroid and the mode of action resulting in tumors have been well described for the rat using prototypical Car/Pxr inducers such as phenobarbital. Although the early Car/Pxr effects in the mouse thyroid are also well described including hepatic enzyme induction, the subsequent imbalance of thyroid hormones, and follicular cell

proliferation following phenobarbital exposure (Qatanani *et al.*, 2005; Pascussi *et al.*, 2008) there are, to our knowledge, no published data of the long-term impact in the mouse. However, considering the data in short-term studies in this species, one can anticipate that phenobarbital has the potential to induce thyroid tumors similar to those observed in the rat.

The Car/Pxr-mediated thyroid tumor induction is a continuum of effects consisting of an initial activation of liver Car/Pxr receptors resulting in an increase in the metabolic capacity of the liver causing an increase in T_4 clearance (due particularly to induction of Phase II metabolic enzymes), which results in a compensatory increase in serum TSH and TSH-dependent stimulation of thyroid gland function and thyroid follicular cell proliferation (Dellarco *et al.*, 2006; McClain, 1992; McClain *et al.*, 1988).

Table 14. Key Events for Car/Pxr-Mediated Thyroid MOA (based on Dellarco et al., 2006)

(1) Car/Pxr receptor activation as evidenced by induction of Phase I liver metabolic enzymes. Reversibility upon discontinuance of treatment
(2) Increased serum T_4 clearance due to induction of Phase II liver metabolic enzymes. Reversibility upon discontinuance of treatment
(3) Increased TSH. Reversibility upon discontinuance of treatment
(4) Increased thyroid cell proliferation; Reversibility upon discontinuance of treatment

During the course of this mechanistic research program it has been a real challenge to evaluate with precision plasma hormone levels (T_4, TSH). However evidence of decreased plasma T_4 was detected in several studies (SA 07215, SA 10241, SA 10430) and a weak increase in TSH was also apparent in a number of studies (SA 0721, SA 10430). This difficulty in evaluating with precision plasma hormone levels is likely a reflection of the weak ability of fluopyram to perturb the pituitary-thyroid axis (i.e. necessity to treat for 12 months before detecting thyroid histopathological changes and only benign tumors were observed in just a few animals (7/50) at the end of the bioassay). In addition technical issues such as imprecision in dose levels received and timing of blood sampling following dietary administration compared to gavage may also have contributed to the difficulty in assessing plasma hormone levels. To get around this difficulty of assessing directly TSH plasma levels it was decided to measure pituitary Tsh

transcript levels as a biomarker that reflects the stimulation of the pituitary to secrete this particular hormone (SA 10241).

In a study designed to confirm the hypothesis of liver mediated thyroid changes fluopyram was evaluated in genetically engineered mice lacking the Car/Pxr nuclear receptors (Pxr/Car KO mice). All critical key events leading to thyroid hyperplasia and adenomas were investigated in this study (Phase I and Phase hepatic enzyme activity, Tsh transcript levels and thyroid follicular cell proliferation); however T4 was not measured due to the absence of a reliable surrogate marker for this hormone. This KO mouse study was considered critical to exclude a direct effect of fluopyram on the thyroid since in the case of a direct effect on the thyroid, both KO and wild type mice would have responded similarly to fluopyram treatment. More specifically, increased pituitary Tsh transcripts and increased thyroid follicular cell proliferation would have been observed in both the wild type and KO mice because the role played by the CAR/PXR nuclear receptors in the thyroid effects would have been inexistent.

Key Event #1: Liver Car/Pxr activation with induction of Phase I metabolic enzymes

The first key event is activation of Car/Pxr nuclear receptors and, as described in an earlier section, evidence for this first key event is generally provided by increased expression of specific hepatic cytochrome P450 genes and activation of their associated enzyme products. In particular, activation of Car/Pxr is associated with the induction of the Cyp2b (Car) and Cyp3a (Pxr) families (Ueda *et al.*, 2002) and their corresponding enzymes as measured by pentoxyresorufin-O-depentylation (PROD) and benzyloxyresorufin-O-debenzylation (BROD) or benzylquinoline (BQ), respectively.

In the mouse, the enzymes specific for Car and Pxr are clearly induced at 30 ppm and higher (Table 15). Activity levels for these enzymes returned to essentially control levels when treatment at the tumorigenic dose level of 750 ppm was followed by a 28-day recovery period on a control diet. Confirmation of Car/Pxr activation was established by comparing WT and Pxr-Car-KO mice; PROD was increased 1.4-fold in KO mice compared to 69.8-fold in WT mice, whereas BQ activity was reduced 1.5-fold in KO

mice compared to an induction of 5.5-fold in WT mice (Table 16). These results clearly show the link between Car/Pxr activation and subsequent induction of Phase I enzymes.

Table 15. Enzyme activity given as fold change examining Phase I (PROD and BQ) enzyme activity from male C57BL/6J mice exposed to fluopyram in the diet for 28 days (N=14-15).

Mouse	Fluopyram - 28 days					
Dose-level (ppm)	30	75	150	600	750	Recovery 750
PROD	**14**	**34**	**37**	**43**	**47**	1.1
BQ	**1.4**	**2.2**	**2.9**	**5.2**	**6.2**	NC

Data from CXR report CXR1284. Bold represents statistically significant differences from the relevant controls. NC: no change from control levels

Table 16. Enzyme activity given as fold change examining Phase I (PROD and BQ) enzyme activity in male C57BL/6J (wildtype) and Pxr-Car knockout (KO) mice exposed to fluopyram in the diet for 28 days.

Mouse	Wildtype	Pxr-Car-KO
Dose (ppm)	750	750
PROD	**69.8**	**1.4**
BQ	**5.5**	**-1.5**

Values are fold change compared to control. N = 14-15. Data from BCS report SA 12162. Bold represents statistically significant differences from the relevant controls.

CONCLUSIONS FOR KEY EVENT 1 (Car/Pxr receptor activation with induction of Phase I liver metabolic enzymes):

- Key Event 1 for mouse thyroid tumor MOA has been identified.
- Dose responses at 30 ppm and higher have been established in male mice for hepatic enzymes associated with activation of Car/Pxr nuclear receptors.
- Induction of these enzymes is reversible following cessation of treatment.
- In the absence of Car/Pxr nuclear receptors, little to no induction of Phase I enzymes occurs.

Key Event #2: Phase II liver enzyme induction leading to increased serum T_4 clearance and consequently decreased circulating T_4

Following Car/Pxr activation, the MOA for thyroid toxicity includes the induction of liver metabolic enzymes that conjugate and eliminate thyroid hormones. T_3 and T_4 are inactivated in the liver mainly by UGT to glucuronide derivatives, which are eliminated via urine and bile (Rutgers *et al.*, 1989) resulting in reduced serum T_4 concentrations in both mice (Hood *et al.*, 2003) and rats (Hood *et al.*, 1999; Liu *et al.*, 1995). The activation of these Phase II enzymes are directly responsible for producing the cascade of lower serum T_4, increased TSH, elevated thyroid follicular cell proliferation, and eventually thyroid tumors (Barter and Klaassen, 1992; Hurley *et al.*, 1998; Dellarco *et al.*, 2006). UGT-T4 and UGT-BIL enzyme activities are known to measure similar if not identical UGT protein families and both were evaluated in the mouse mechanistic studies.

A dose-related increase in the activity of both isoforms of UGT starting from 150 ppm was recorded following treatment of the mice with fluopyram for 28 days, which was fully recovered following a 28 day recovery period (Table 17). It is likely that the absence of a statistically significant response for UGT-T4 at 750 ppm is due to assay variability, because in an independent study of an identical design, a 1.8-fold increase (statistically significant) in UGT-T4 activity was observed in WT mice (Table 18). In contrast, the activity measured in Pxr-Car-KO mice was essentially comparable to the untreated control group.

Table 17. Enzyme activity given as fold change examining Phase II (UGT-T4 and UGT-BIL) enzyme activity from male C57BL/6J mice exposed to fluopyram in the diet for 28 days (N=14-15).

Mouse	Fluopyram - 28 days					
Dose-level (ppm)	30	75	150	600	750	Recovery 750
UGT-T4	1.0	1.1	*1.5*	**1.8**	*1.3* (**1.8**)[A]	1.1
UGT-BIL	1.1	1.2	*1.3*	**1.4**	**1.5**	-1.1

Data from BCS report SA 11105. Bold represents statistically significant differences from the relevant controls. Data in italics considered biologically significant in the absence of statistics.
[A] A significant increase in Ugt-T4 at 750 ppm fluopyram was recorded in a separate study (see Table 18).

Table 18. Enzyme activity examining Phase II (UGT-T_4 and UGT-BIL) enzyme activity in male C57BL/6J (wildtype) and Pxr-Car knockout (KO) mice exposed to fluopyram in the diet for 28 days.

Mouse	Wildtype	Pxr-Car KO
Dose (ppm)	750	750
UGT-T_4	**1.8**	1.2
UGT-BIL	**1.8**	-1.1

Values are fold change compared to control. N = 14-15. Data from BCS report SA 12162. Bold represents statistically significant differences from the relevant controls.

These hepatic Phase II enzyme activity data provide evidence that fluopyram induces specific UGT enzyme activities at ≥150 ppm and that Car/Pxr activation is obligatory for this enzyme activity induction. The activation of Phase II UGTs is also seen with other Car/Pxr-inducing chemicals that lead to rodent thyroid tumors (McClain, 1992; Hurley *et al.*, 1998).

Two independent but limited T_4 clearance studies were conducted in male mice using radioactive T_4. The results showed that pre-treatment with 2000 ppm fluopyram for 3-4 days resulted in a clear increased clearance of T_4 at the earliest time point measured, when compared to control mice (Fig 1). Similar results were seen for phenobarbital. Overall the data provide evidence that the decrease of plasma T_4 induced by fluopyram cannot be due to a decreased biosynthesis and release of thyroid hormones. Moreover, these data are supported by an *in vitro* study (Bayer HealthCare report AT 04481), which showed that fluopyram does not interfere with the activity of a key enzyme involved in the synthesis of thyroid hormones (ie. thyroid peroxidase (TPO)).

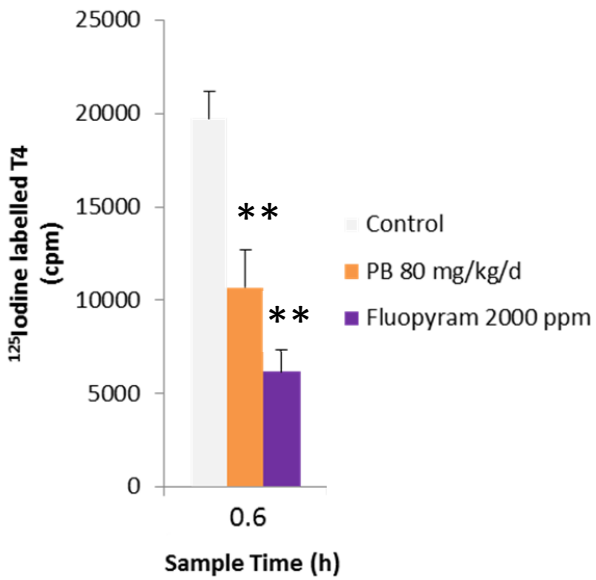


Figure 1. Data is from BCS report SA 08288. Mean (±SD) whole-blood radioactivity concentrations on three groups of male mice (8 animals per group) administered with single intravenous injections of ^{125}I-thyroxine after 4 days pre-treatment with control diet, fluopyram 2000 ppm or phenobarbital 80 mg/kg/day. **p≤0.001

Several studies were conducted in the mouse to determine the effects of fluopyram treatment on circulating thyroid hormone levels. A significant decrease in T_4 was observed as early as three days in male mice exposed to 2000 ppm fluopyram (Table 19), consistent with a perturbation of thyroid hormone homeostasis that results from increased T_4 clearance. Additional evidence for increased T_4 clearance was provided in a separate three day study at 2000 ppm fluopyram, where clear and significant increases in transcript levels for UGT as well as sulfotransferases were recorded (SA 08151).

Table 19. Thyroid hormone levels (T_3 and T_4) reported as percent (%) change compared to controls in male C57BL/6J mice exposed to 2000 ppm fluopyram for 3 or 14 days.

Male mice	% change compared to control	
Group	3 day	14 day
T_3	1	5
T_4	**-30**	**-27**

Data is from BCS report SA 07215. Bold represents statistically significant differences from the relevant controls.

CONCLUSIONS FOR KEY EVENT 2 (Phase II liver enzyme induction leading to increased serum T_4 clearance and consequently decreased circulating T_4):

- Key event 2 for thyroid tumor MOA has been identified.
- Dose responses have been established and thresholds of response at ≥ 150 ppm identified for Phase II enzymes known to be involved in T_4 clearance.
- The changes in Phase II enzyme activity are reversible following cessation of fluopyram
- Activation of Car/Pxr nuclear receptors is obligatory to observe phase II enzyme induction by fluopyram as demonstrated in the Car-Pxr-KO mouse study.
- There is evidence of increased T_4 clearance and decreased plasma T_4 levels.

Key Event #3: Increased TSH

The first two key events leading to Car/Pxr-induced thyroid alterations occur in the liver; however, reduced circulating levels of T_4 can activate the hypothalamic-pituitary-thyroid axis feed-back mechanism in an attempt to maintain thyroid hormone homeostasis (Rapoport and Spaulding, 1996). Serum T_4 and T_3 concentrations are monitored by the hypothalamus and the anterior pituitary gland. A decrease in serum thyroid hormone concentrations stimulates the hypothalamus to secrete TRH, which then stimulates the release of TSH from the anterior pituitary. In addition to TRH, the secretion of TSH from the anterior pituitary is also regulated by circulating thyroid hormone concentrations (Scanlon and Toft, 1996). Increased pituitary secretion of TSH stimulates the thyroid gland to produce more T_4 and T_3 to restore thyroid hormone homeostasis.

Due to difficulties in detecting clear changes in TSH plasma levels in early studies (SA 10241, SA 10430) it was decided to measure pituitary "thyroid- stimulating hormone, beta" (Tsh *b*) transcript levels in the later studies. *Tsh b* is a gene that provides instructions for making the specific protein subunit of TSH, a hormone designed to stimulate the function and the growth of the thyroid gland resulting in an increased production of thyroid hormones. When thyroid hormone levels are low, the pituitary gland releases TSH into the bloodstream, which stimulates the thyroid gland to produce

more thyroid hormones. Thus, an increase in *Tsh b* can serve as a biomarker for increased TSH levels.

In a 28-day study examining *Tsh b* in mice, a significant induction of this transcript was observed in the pituitary gland at 600 and 750 ppm fluopyram (Table 20).

Table 20. Measure of thyroid stimulating hormone, beta (*Tsh b*) gene transcript from the pituitary gland in male C57BL/6J mice exposed to fluopyram for 28 days.

Mouse	Fluopyram					
Dose (ppm)	30	75	150	600	750	750 recovery
Tsh b	1.1	1.1	1.1	**1.4**	**1.5**	1.1

Results are given as fold change compared to control. Bold represents statistically significant differences from the relevant controls. Data from BCS report SA 11105.

Following cessation of treatment for 28 days, the levels of this transcript returned to normal levels.

Confirmation that Car/Pxr activation is required to produce changes in *Tsh b* transcript levels is provided when examining the gene expression of *Tsh b* in Pxr-Car-KO mice treated with fluopyram, as no change was seen in transcript levels in these mice (Table 21). This result provides further evidence that without the activation of Car/Pxr followed by increased UGT enzyme activity, there is no perturbation of the pituitary-thyroid axis.

Table 21. Measure of *Tsh b* gene transcript from the pituitary gland of wildtype and Pxr-Car knockout (KO) male mice exposed to dietary fluopyram for 28 days.

Mouse	Wildtype	Pxr-Car KO
Gene transcript	750 ppm	750 ppm
Tsh b	**1.6**	-1.1

Bold represents statistically significant differences from the relevant controls. Data from BCS report SA 12162.

CONCLUSIONS FOR KEY EVENT 3 (*Increased TSH*):

- Key Event 3 for thyroid tumor MOA has been identified.
- Dose response and a threshold of response have been identified for the *Tsh b* gene transcript (responsible for making a protein subunit of TSH) at ≥ 600 ppm.
- The changes associated with Key Event 3 are reversible following cessation of fluopyram.
- *Tsh b* gene transcript induction did not occur in Car-Pxr-KO mice demonstrating the obligatory activation of liver Car/Pxr nuclear receptors by fluopyram to observe treatment-related *Tsh b* gene transcript changes.

Key Event #4: Increased thyroid cell proliferation

Chronic elimination of T_4 and T_3 by a liver metabolically induced by exposure to a Car/Pxr activator requires the thyroid gland to increase production of these hormones. The demand for increased thyroid hormones by TSH is met by increased thyroid follicular cell proliferation (Hood *et al.*, 1999; Hood *et al.*, 2003). This process of thyroid follicular cell proliferation, if sustained for a prolonged period of time, can result in thyroid gland tumors (McClain, 1992). The hypothesis of sustained proliferation leading to tumorigenesis is similar to that described for the liver.

In a dose-response study in mice, an increase in thyroid follicular cell proliferation was seen starting from 150 ppm (Table 22). This effect was reversible after treatment at 1500 ppm followed by 28 days on a control diet. In WT and Car-Pxr-KO mice treated at 750 ppm, a significant increase was observed in the thyroid gland proliferation index for WT mice, whereas the treated KO mouse group was similar to the KO control (Table 23). The absence of increased cellular proliferation in KO mice demonstrates that the activation of the Car/Pxr receptor is obligatory for the induction of the thyroid follicular cell alterations in mice exposed to fluopyram.

Table 22. Thyroid follicular cell proliferation index expressed as mean ± SD in C57BL/6J male mice exposed to fluopyram for 28 days.

Mouse	Males 28 days (N = 14 – 15)							
Dose (ppm)	0	30	150	600	750	1500	Control recovery	1500 ppm recovery
Thyroid Gland proliferation index	21.55± 4.75	17.81 ± 7.37	*26.09 ± 8.62*	**30.11 ± 8.53**	**34.78 ± 7.61**	**50.21± 10.24**	17.57± 5.18	11.56± 4.78

Bold represents statistically significant differences from the relevant controls. Data is from BCS study SA 12058. Italics considered biologically relevant but not statistically significant.

Table 23. Thyroid follicular cell proliferation index in wild-type and Car/Pxr knock-out male mice exposed to fluopyram for 28 days expressed as mean ± SD.

Mouse	Wildtype Control	Wildtype 750 ppm	Pxr-Car KO Control	Pxr-Car KO 750 ppm
Thyroid Gland Proliferation Index	14.28 ± 3.96	**26.08 ± 7.16**	10.05 ± 3.88	9.91 ± 4.06

Bold represents statistically significant differences from control. Data is from BCS study SA 12162.

Sustained cellular proliferation resulted in a low incidence (20-22%) of thyroid follicular cell hyperplasia in male mice at 150 and 750 ppm after 12 months of treatment. After 18 months, the incidence was 2 to 3 times higher in these treatment groups compared to the 12 month interval (Table 24).

CONCLUSIONS FOR KEY EVENT 4 (*Increased thyroid follicular cell proliferation*):

- Key Event 4 for thyroid tumor MOA has been identified.
- Dose responses have been established and a threshold of effect identified from 150 ppm for follicular cell proliferation.
- Follicular cell proliferation was reversible on cessation of treatment.
- Follicular cell proliferation did not occur in Car-Pxr-KO mice demonstrating the obligatory activation of liver Car/Pxr nuclear receptors by fluopyram to observe the treatment-related thyroid follicular cell proliferation.

Key Event #5: Increased thyroid cell hyperplasia

In male mice exposed to 2000 ppm fluopyram for 3 or 14 days, no evidence of macro- or microscopic changes to the thyroid gland was observed (SA 07215). Furthermore, no adverse thyroid findings were noted in the 28- and 90-day mouse studies at up to 5000 ppm and 1000 ppm, respectively. Thyroid follicular cell hyperplasia was observed at ≥ 150 ppm in males at 12 and 18 months and at 750 ppm in females at 18 months. As the microscopic effects induced in the thyroid by fluopyram were observed only after 12 and 18 months of treatment, fluopyram shows a weak ability to produce thyroid effects.

Table 24. Thyroid follicular cell hyperplasia in the male C57BL/6J mouse exposed to fluopyram for 12 or 18 months.

Mouse		Males			
	Dose (ppm)	0	30	150	750
12 months (N=9-10)	Follicular cell hyperplasia (%)	0	0	*22*	*20*
18 months (N=48-50)	Follicular cell hyperplasia (%)	8	12	**42**	**64**

Bold represents statistically significant differences from the relevant controls. Italics considered biologically significant in the absence of statistics. 12 and 18 month mouse data is from BCS report SA 05094.

CONCLUSIONS FOR KEY EVENT 5 (*Increased thyroid follicular cell hyperplasia*):

- Key Event 5 for thyroid tumor MOA has been identified.
- Dose responses have been established and a threshold of effect identified at ≥150 ppm for follicular cell hyperplasia.
- Chronic administration demonstrated sustained follicular cell proliferation as observed by increased levels of follicular cell hyperplasia at 12 and 18 months.

Mouse Thyroid Associative Events

Increased liver and thyroid weight, microscopic hepatocellular and follicular cell hypertrophy (in the rat), and reversibility on discontinuance of treatment have been identified as associative events for Car/Pxr-induced thyroid changes.

The inclusion of increased liver weight and hypertrophy within the thyroid associative events for Car/Pxr-induced thyroid tumors signify the key role that the liver plays in the

formation of these tumors. Within the standard 28 and 90 day mouse studies, significant findings of increased liver weight and hepatocellular hypertrophy were seen ≥ 150 ppm. Furthermore, when comparing the liver microscopic findings in male and female mice, the data show that fluopyram induced liver effects in males are greater than in females. In the cancer bioassay, centrilobular to panlobular hepatocellular hypertrophy was higher in incidence and in severity in males at 750 and 150 ppm, when compared to the female findings (Table 25). Hepatocellular hypertrophy is considered as an enlargement of cytoplasm which may be a consequence of endoplasmic reticulum proliferation. This finding is associated with the presence of multinucleated hepatocytes at 750 ppm only in males. Finally, in the 90- day toxicity study in mice, hepatocellular hypertrophy was more severe in males than in females at 150 or1000 ppm. Overall, these data provide evidence that the potent liver induction in males is likely to be associated with the thyroid tumor formation, reinforcing that fluopyram induces thyroid tumors indirectly via the liver Car/Pxr activation.

Table 25. Liver microscopic changes observed in the mouse cancer bioassay

Incidence and severity of microscopic changes in the liver, all animals, carcinogenicity phase								
Sex	Males				Females			
Dose level of fluopyram (ppm)	0	30	150	750	0	30	150	750
Number of animals	49	49	49	50	48	50	50	50
Centrilobular to panlobular hepatocellular hypertrophy: diffuse								
Minimal	0	0	16	3	0	0	18	24
Slight	0	0	22	11	0	0	0	2
Moderate	0	0	0	36	0	0	0	0
Total	**0**	**0**	**38**	**50**	**0**	**0**	**18**	**26**
Multinucleated hepatocytes: focal/multifocal								
Minimal	3	1	3	25	1	0	0	0
Slight	0	0	1	2	0	0	0	0
Total	**3**	**1**	**4**	**27**	**1**	**0**	**0**	**0**

Data is taken from BCS report SA 05094, following 18-months of treatment

Generally, effects on liver enzymes/weight and pituitary-thyroid hormone concentrations would be anticipated to occur at doses at least as low as those that produce thyroid weight changes and increases in thyroid tumor incidence, given that this thyroid disruption MOA action is a threshold phenomenon (Dellarco *et al.*, 2006). The Society of Toxicologic Pathology recommends that thyroid gland weights be gathered routinely in all species except mice (Sellers *et al.*, 2007). Weighing the thyroid gland in mice should be optional because the act of collection and weighing may produce artifacts that can complicate or even prevent microscopic assessment. Furthermore, in the OPPTS Guideline 870.4200 for conduct of a carcinogenicity study in rodents, the thyroid is not a required tissue to be weighed. Because of these limitations and to ensure the best quality tissue for pathological examination, thyroid weight was not measured in the mouse.

In the absence of mouse thyroid follicular cell hypertrophy, the rat data at 12 and 24 months can be examined, principally within the high dose groups. These data show a consistent pattern of altered thyroid hormone homeostasis leading to pathologically altered thyroid glands in both the mouse and rat.

Rat Supportive Data

Although the tumors and primary mechanistic data informing the MOA for thyroid tumors were in the mouse, additional information can be gleaned from studies conducted in the rat. Below is a summary of the liver metabolic enzymes that play a role in thyroid hormone elimination.

Rat gene *Udpgr2*, recognized for possible involvement in removing T_4 from the circulation and eliminating it in the bile, was significantly elevated consistently at ≥ 600 ppm (Table 26) and on cessation of treatment at 1500 ppm, returned to control levels. Furthermore, thyroid-associated enzyme activity in the rat, as measured by UGT-BIL, was significantly elevated at ≥ 150 ppm (Table 27).

Table 26. Gene expression in female Wistar rats of liver *Udpgtr2*, a gene known to play a role in the enzymatic process of thyroid hormone elimination.

Rat Gene *Udpgtr2* (fold-change relative to controls)					
Dose (ppm)	30	75	150	600	1500
3 days	1.1	1.1	1.7	**2.4**	**3.8**
7 days	1.4	**1.5**	1.4	**2.9**	**4.7**
28 days	1.2	1.2	**1.6**	**2.5**	**4.0**
28 days followed by 28 days recovery	-	-	-	-	0.78

Bold represents statistically significant differences from the relevant controls. Three and 7 day data are from BCS report SA 10240, and the 28 day data are from report SA 11104.

Table 27. Enzyme activity in female Wistar rats of liver UGT-bilirubin known to play a role in thyroid hormone elimination.

Rat Fluopyram dose (ppm)	Mean Fold Change Relative to Controls
7 days	UGT-BIL
30	1.1
75	1.3
150	1.4
600	**1.9**
1500	**2.7**
28 days	UGT-BIL
30	1.1
75	1.2
150	**1.6**
600	**2.1**
1500	**2.8**
28 days followed by 28 days recovery	UGT-BIL
1500	**1.3**

Bold represents statistically significant differences from the relevant controls. Data from BCS reports SA 10240 (7 days) and SA 11104 (28 days).

In male and female rats exposed to 0, 50, 200, 1000, and 3200 ppm fluopyram, TSH was measured at 21 and 90 days. In that study (SA 04048), a significant increase in TSH was seen in the high-dose males (1.6-fold) and females (1.7-fold) at 21 days and in males (1.9-fold) at 90 days.

Rats exposed to fluopyram had significantly elevated thyroid gland weights and follicular cell hypertrophy and hyperplasia at the dose that also induced liver tumors (females only) (Table 28). These data provide further evidence that activation of Car and Pxr by fluopyram results in alterations to both the liver and thyroid in the mouse and rat.

Table 28. Incidence of microscopic changes given as % change compared to control or % occurrence in the thyroid gland from Wistar rats treated with fluopyram for 12 (N = 10) or 24 months (N = 54 - 60).

	Rat	Males				Females			
12 months	Dose (ppm)	0	30	150	750	0	30	150	1500
	Mean liver to bw ratio (% change)	0	4	1	**17**	0	1	**4**	**54**
	Mean thyroid gland to bw ratio (% change)	0	3	-10	9	0	-1	9	**38**
	Follicular cell hypertrophy (% occurrence)	0	0	*20*	*40*	0	0	0	*50*
24 months	Dose (ppm)	0	30	150	375	0	30	150	1500
	Mean liver to bw ratio (% change)	0	-6	8	12	0	-3	6	**56**
	Mean thyroid gland to bw ratio (% change)	0	5	-3	7	0	-2	6	**10**
	Follicular cell hypertrophy (% occurrence)	2	0	7	**13**	0	0	2	**21**
	Follicular cell hyperplasia (% occurrence)	1	0	1	**3**	2	2	1	**4**

Data from BCS report SA 04312. Bold represents statistically significant differences from the relevant controls. Italics considered biologically significant in the absence of statistics.

Summary of Fluopyram Thyroid Tumor MOA

The MOA for fluopyram-induced rodent thyroid effects is similar to that for known Car/Pxr inducers. The use of Car/Pxr knockout mice has demonstrated that fluopyram is a Car/Pxr inducer producing typical liver effects and thyroid alterations. The relevant molecular and pathological endpoints for fluopyram-induced liver and thyroid effects are summarized below. This analysis is based on the mechanistic and standard repeat-dose toxicity studies in mice administered fluopyram and show that the data 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 as observed by increases in the Phase I enzymes PROD (specific for Car) and BQ (specific for Pxr). On cessation of treatment, enzyme activities returned to control levels. As expected in Pxr-Car-KO mice, only marginal 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. 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 exposure, 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, 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. This finding shows that activation of Car and Pxr in the liver is obligatory to induce thyroid alterations following exposure to fluopyram.

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. 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. The effect was observed at ≥ 150 ppm.

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. 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.

3. Strength, Consistency, and Specificity of Association of Tumor Response with Key Events

In applying the Bradford-Hill criteria to the aforementioned fluopyram MOA data, one must demonstrate that the key events are causally related to the formation of tumors, the key events are actually required steps that lead to tumors, and these data are reproducible. The key events observed following exposure to fluopyram in both the mouse and rat occurred in a biologically relevant temporal sequence, were dose-dependent, and took place at dose levels that were at or below the doses that produced tumors.

Liver tumor MOA—The key events for fluopyram-induced rat liver tumor MOA were defined as: 1) activation of Car/Pxr, 2) increased hepatocellular proliferation, and 3) increased altered hepatic foci. All of these key events were successfully identified, key events 1 and 2 were shown to be reversible, and all had a clear dose- and temporal-response.

Thyroid tumor MOA—The key events for fluopyram-induced mouse thyroid tumors were defined as: 1) activation of Car/Pxr, 2) induction of UGT (with an indication for increased T_4 clearance and decreased plasma T_4 levels), 3) increased TSH (*Tsh β*), 4) increased thyroid follicular cell proliferation, and 5) increased thyroid follicular cell hyperplasia. All of these key events were successfully identified and characterized in terms of dose and temporal response, and were shown to be reversible. Importantly, key events 1 – 4 were absent in Car-Pxr-KO mice exposed to 750 ppm fluopyram (Table 29) which demonstrates that the liver Car /Pxr activation is the initial molecular event causing the thyroid tumors in male mice .

Table 29. Comparison of fluopyram induced in wild-type and Pxr-Car knockout mice

Male mice		750 ppm fluopyram/28 days dietary	
Corresponding Key Event	Parameter	Wild-type	Pxr-Car-KO
1	Hepatic Phase I enzyme activity	↑ PROD/BQ	No change
2	Hepatic Phase II enzyme activity	↑UGT-T4/UGT-BIL	No change
3	Tsh β	↑	No change
4	Thyroid follicular cell proliferation	↑	No change

Data taken from BCS report SA 12162

Overall, the data informing the MOA of fluopyram provide a strong and consistent association with the MOA known to induce liver and thyroid tumors in rodents following exposure to Car and Pxr inducers.

4. Biological Plausibility and Coherence.

Induction of CYP enzyme activity is a well-known mechanism involved in rodent hepatocarcinogenesis, with phenobarbital as a standard example (Whysner *et al.*, 1996; Holsapple *et al.*, 2006). Phenobarbital is non-genotoxic and considered a tumor promoter in rodents. The key events for this MOA include activation of Car leading to CYP enzyme induction, increased hepatocellular proliferation, and subsequent induction of proliferative lesions in the liver, including hepatocellular foci, adenomas, and carcinomas (Cohen, 2010). There is uncertainty as to whether the CYP induction is a necessary, critical step or an indicator of chemical activity that is secondary to activation of Car and Pxr (associative). Activation of rodent Car and Pxr produces a cascade of alterations in gene transcription that leads to increased hepatocellular proliferation in rodents, a critical event in the development of liver tumors (Whysner *et al.*, 1996; Cohen, 2010).

The important role of Car in phenobarbital-induced rodent liver tumor formation has been demonstrated in genetically engineered mice lacking this nuclear receptor (reviewed in Lake, 2009). In Car-KO mice, phenobarbital exposure does not induce Cyp2b forms or liver enzyme activity or stimulate replicative DNA synthesis (Wei *et al.*, 2000; Huang *et al.*, 2005). In addition, although no liver tumors were observed in Car-KO mice following initiation with diethylnitrosamine and promotion with phenobarbital, they were seen in WT mice (Yamamoto *et al.*, 2004). Further, because of frequent involvement of Pxr following exposure to known Car inducers (Nakata *et al.*, 2006; Moreau *et al.*, 2007), a mouse model was developed in which both Car and Pxr are absent (Pxr-Car-KO) (Ross *et al.*, 2010). These KO mice models were developed in an attempt to eliminate confounding issues of receptor cross-talk and aid in identifying the role of these receptors to adverse changes following chemical exposure.

The effects on the thyroid and the mode of action resulting in tumors have been well described for the rat using prototypical Car/Pxr inducers such as phenobarbital (Hiasa *et al.*, 1982; McClain *et al.*, 1988). The early Car/Pxr effects in the mouse thyroid are also well described and include hepatic enzyme induction, the subsequent imbalance of

thyroid hormones, and follicular cell proliferation following phenobarbital exposure (Qatanani *et al.*, 2005). Furthermore, using wild-type and Car KO mice, Qatanani *et al.* (2005) demonstrated phenobarbital decreased serum T4, increased serum TSH, and induced gene expression of several UGTs in wild-type but not Car KO mice. Dietary administration of fluopyram to mice and rats results in the key events (Car/Pxr activation and hepatocellular/ thyroid follicular cell proliferation) that result in hepatocellular and thyroid tumors after prolonged exposure to high-dose levels of fluopyram (1500 ppm in rats and 750 ppm in mice). The early key events of hepatic enzyme induction and cellular (liver and thyroid) proliferation as well as the associative events of increased liver weight and hepatocellular hypertrophy are reversible on cessation of treatment.

Additional support for the MOA was given by using a genetically engineered mouse model and a comparative rat/human hepatocyte assay. As previously described, Pxr-Car-KO mice were refractory to the Car/Pxr-mediated hepatic and thyroid effects demonstrated for fluopyram in WT mice.

The data for fluopyram support a direct, threshold-based, dose-responsive MOA for liver tumors in the rat and thyroid tumors in the mouse. The MOA demonstrated for fluopyram is consistent with the well-understood MOA for Car and Pxr inducers (Whysner *et al.,* 1996; Holsapple *et al.,* 2006; Cohen, 2010; Hurley *et al.*, 1998; Dellarco *et al.*, 2006; Qatanani *et al.*, 2005), and it is consistent with the current understanding of cancer biology and nuclear-receptor mediated carcinogenesis. The data for fluopyram are consistent with this non-genotoxic MOA in the liver and thyroid.

The data for fluopyram are judged with a <u>high degree of confidence</u> to adequately explain the development of liver tumors in the female rat and thyroid tumors in the male mouse following chronic dietary exposure.

Table 28.

Analysis of Fluopyram Rat Liver Tumor MOA

Key Event #1: Car/Pxr receptor activation w/ associated CYP enzyme induction w/ associated liver hypertrophy Key Event #2: Hepatocellular proliferation KeyKey Event #3: Altered hepatic foci Key events 1 and 2 were shown to be reversible.	
Strength of association	+
Consistency of association	+
Specificity of association	+
Dose-response concordance	+
Temporal relationship	+
Coherence & plausibility	+ Plausible; + Coherence

Analysis of Fluopyram Mouse Thyroid Tumor MOA

Key Event #1: Car/Pxr receptor activation w/ Associated CYP enzyme induction Key Event #2: Phase II liver enzyme induction and decreased T4 Key Event #3: Increased TSH Key Event #4: Thyroid follicular cell proliferation All of these key events are reversible.	
Strength of association	+
Consistency of association	+
Specificity of association	+
Dose-response concordance	+
Temporal relationship	+
Coherence & plausibility	+ Plausible; + Coherence

5. Other MOAs.

As listed in Table 29, the MOAs for liver tumorigenesis are broadly categorized as requiring increased cell proliferation through either receptor or non-receptor mediated processes or DNA reactivity (Cohen, 2010). The MOAs for liver tumorigenesis also have a corollary to thyroid tumorigenesis in that some of the biological responses given for the liver hold true for the thyroid *e.g.,* DNA reactivity.

Table 29. Modes of Action for Hepatocellular Carcinogenesis (Cohen, 2010).

- **I. DNA reactivity**
- II. Increased cell proliferation
  - a. Receptor mediated
    - i. Ppar (peroxisome proliferation)
    - ii. Enzyme induction (Car, Pxr, AhR)
    - **iii. Estrogen**
    - iv. Statins
    - **v. Cytotoxicity**
    - vi. Other
  - b. Non-receptor mediated
    - **i. Cytotoxicity**
    - **ii. Infectious**
    - **iii. Iron (copper) overload**
    - iv. Increased apoptosis (e.g., fumonisin B1)
    - v. Other

Modes of action in bold letters are likely to be relevant to humans.

DNA reactivity. DNA reactivity is the first broad category of an 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—all conducted in the absence or presence of a metabolic activation system (rat liver S9), showed that fluopyram does not cause gene mutations or chromosome aberrations. Additionally, an *in vivo* mouse micronucleus assay showed that fluopyram does not induce micronuclei in somatic cells. An evaluation of the genetic toxicity data for fluopyram unequivocally supports that DNA reactivity is not a potential MOA for the induction of tumors in mice or rats.

Receptor-mediated, increased cell proliferation. MOAs for hepatocellular carcinogens that cause receptor-mediated hepatocellular proliferation include AhR, Car, Pxr, and Pparα, as well as estrogens and statins (Cohen, 2010). The MOA studies in mice and rats with fluopyram clearly demonstrate a specific, dose-related increase in the *Cyp2b*/Car-associated and *Cyp3a*/Pxr-associated gene and enzyme activity. Furthermore, Pxr-Car-KO mice supported specificity for fluopyram-induced activation of Car and Pxr. Taken together, these findings are consistent with direct activation of both Car and Pxr nuclear receptors. At the same time, the MOA studies in mice and rats excluded receptor (Pparα, AhR, ER, statin, and cytotoxicity) and non-receptor-mediated (cytotoxicity, infections, iron overload, and increased apoptosis) involvement in the key events leading to fluopyram-induced cancers as discussed below.

Activation of the Proliferator-Activated Receptor Alpha (Pparα). Pparα belongs to the superfamily of nuclear receptors that can be activated by fatty acids and their metabolic derivatives. Pparα has been associated with the regulation of lipid metabolism, glucose homeostasis, cellular differentiation, cancer development, and inflammation (Chinetti *et al.*, 2000; Desvergne and Wahli, 1999; Duval *et al.*, 2002). In mice exposed to the prototypical Pparα inducers WY-14643 and clofibrate, *Cyp4a10* transcripts were induced 6.2- and 4.6-fold, respectively (Takeuchi *et al.*, 2006; Wang *et al.*, 2008). Furthermore, a study examining clofibrate exposure to rats for 4 days reported 18-fold elevated *Cyp4a1* mRNA (Konig *et al.*, 2007). However, the Car/Pxr activator phenobarbital, reduced Pparα-related gene and enzyme activity in rats (80 mg/kg/d I.P. for 3 days) and mice (100 mg/kg/d I.P single dose) (Baldwin *et al.*, 2006; Ueda *et al.*, 2002; Wang *et al.*, 2008).

To address possible peroxisome proliferation activity by fluopyram, liver *Cyp4a1* RNA levels were examined in the rat. This gene is considered an indicator of Pparα activation (Aldridge *et al.*, 1995). Within the mechanistic studies for fluopyram, the majority of *Cyp4a1* transcript levels were slightly reduced, and only the 7-day 1500 ppm response was statistically identified (-3.2-fold, Table 30). These data show that the Pparα-related transcript *Cyp4a1* is slightly reduced in a similar fashion to that observed for the Car/Pxr activator phenobarbital and signifies that fluopyram is not an agonist or inducer of this

nuclear receptor. Therefore, this receptor would not play a role in the key events leading to fluopyram-induced cancers.

Table 30. Levels of *Cyp4a1* transcript given as fold-change compared to control from female Wistar rats exposed to various doses of fluopyram for 3, 7, or 28 days.

Rat *Cyp4a1*					
Dose (ppm)	30	75	150	600	1500
3 days	-1.1	1.0	-1.1	-1.0	-1.3
7 days	-2.1	-2.4	-2.3	-2.3	**-3.2**
28 days	-1.2	-1.1	-1.0	-1.2	-1.4

Bold represents statistically significant differences from the relevant controls. Three and 7 day data are from BCS report SA 10240 and the 28 day data are from report SA 11104.

Activation of the Aryl Hydrocarbon Receptor (AhR). The AhR is a ligand-activated transcription factor involved in the regulation of several genes, including those for xenobiotic metabolizing enzymes such as cytochrome P450 Cyp1a1/2 (Nebert, 2000). Activators of AhR include a variety of polycyclic aromatic hydrocarbons, including the chlorinated dioxins and related halogenated aromatic hydrocarbons. Fluopyram is not a polycyclic aromatic hydrocarbon as it does not have fused aromatic rings and contains elements other than hydrogen and carbon (nitrogen, oxygen, fluorine, and chlorine). Furthermore, it is not a chlorinated dioxin; however, it can be classified as a halogenated aromatic hydrocarbon molecule because of the presence of fluorine and chlorine. To experimentally investigate activation of this receptor, *Cyp1a1* is used as a sensitive, although non-specific, indicator of AhR binding and activation, whereas EROD is used to examine the activity of this enzyme (Hu *et al.*, 2007). Evaluation of the *Cyp1a1* gene expression levels and EROD liver enzymatic activity from fluopyram-exposed animals is summarized in Table 31.

Table 31. *Cyp1a1* gene expression and EROD enzyme activity expressed as fold change compared to control results from female Wistar rats exposed to fluopyram for up to 28 days with a 28 day recovery group of the top dose.

Rat female					
Cyp1a1	30 ppm	75 ppm	150 ppm	600 ppm	1500 ppm
3 days	-1.2	1.1	1.7	**7.3**	**62.7**
7 days	1.4	1.8	**4.6**	**63.6**	**222.9**
28 days	1.8	2.3	**8.1**	**100.9**	**354.7**
Recovery	–	–	–	–	**1.8** (↓100%)
EROD					
7 days	1.1	0.9	1.0	1.1	**1.6**
28 days	1.1	1.1	**1.3**	**1.3**	**2.0**
Recovery	–	–	–	–	1.2 (↓78%)

Bold represents statistically significant differences from the relevant controls. Three and 7 day data are from BCS report SA 10240 and the 28 day data are from report SA 11104.

Although there was a large increase in *Cyp1a1* transcript at the top dose of 1500 ppm (355-fold) at 28 days, this did not translate into a similar magnitude of increased enzyme activity, as EROD was only elevated 2-fold. In BCS studies examining EROD levels in rats exposed to the known AhR agonist β-naphthoflavone, EROD levels were increased 9.1-fold.

Table 32. 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 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. β-Naphtoflavone data is from BCS study SA 06181. Bold represents statistically significant differences from the relevant controls.

These results show a discordant response with a large induction of *Cyp1a1* gene transcript and a very minimal increase in EROD enzyme activity in the rat following exposure to fluopyram. Rat EROD levels were also consistently lower than those seen for the positive control β-Naphtoflavone.

It has been suggested that increased EROD activity observed following exposure to Car-inducers is primarily seen after a significant induction of Cyp2b enzyme (as measured by PROD and BROD) (Corcos *et al.*, 1998). In mice (14 days) and rats (28 days) exposed to carcinogenic levels of fluopyram, EROD levels were 20- and 35-times lower than those seen for BROD. The consistently lower levels of EROD activity compared to the Car/Pxr-associated enzymes for fluopyram support the observations of Corcos and colleagues. Furthermore, *Cyp1a2*, a Car-inducible gene not regulated by AhR, can also significantly elevate EROD activity (Sakuma *et al.*, 1999). Thus, it is highly likely that some proportion of EROD activity observed following fluopyram exposure was the result of Car induction and/or crosstalk between Car/Pxr and AhR (Moreau *et al.*, 2007). In evaluating these data, it is unlikely that fluopyram is a significant agonist or activator of AhR, and any involvement of this receptor in the key events leading to fluopyram-induced tumors is minimal and most likely due to cross-talk subsequent to the significant induction of Car and Pxr.

Activation of the Estrogen Receptor (ER). Estrogens have a specific receptor-mediated MOA that results in cell proliferation in tissues, including the liver; however, the carcinogenic activity may be due to the result of an interaction of DNA adduct formation with increased cell proliferation (dual MOA) (Cohen, 2010). Fluopyram is not likely to have an estrogenic MOA based on its structural dissimilarity to estrogens. When evaluated for chemical structural similarity using the Tanimoto similarity index, fluopyram is only 8.6% and 8.9% similar to 17-alpha-ethinylestradiol (CAS 77538-56-8) and estradiol (CAS 50-28-2), respectively.

When analyzed using QSAR techniques (QSAR Toolbox version 3.0.0.995) for ER binding and with the “US EPA ER expert system”, fluopyram was found to have the status of “non-binder” and “no alerts found”, respectively. For ER binding, the QSAR

evaluation stated, "Chemicals that have a molecular weight less than 500, and possess a cyclic structure but one without a hydroxyl or amino group are reported to be non-binders to the [estrogen] receptor (Cronin and Worth, 2008; Tong *et al.*, 2004)." Most importantly, in the standard rat and mouse studies and the two-generation rat study, no evidence of interference in the estrogen system was observed, *e.g.,* decreased fertility in males, alterations in male and female reproductive organ weights, estrous cyclicity, precocious vaginal opening. Additionally, fluopyram was not a developmental or reproductive toxicant. The available developmental toxicity studies in rats and rabbits and the multi-generation reproduction study in rats demonstrate no evidence of increased qualitative or quantitative susceptibility in developing or young animals following exposure during pre- or post-natal periods. From these data, it is concluded that fluopyram does not act as an agonist or activate the ER, and thus this receptor would not play a role in the development of fluopyram-induced cancers.

Statins. The rodent profile for a statin-induced liver tumor MOA consists of an increase in liver HMG-CoA-reductase, *Cyp2b* and *Cyp4a* transcript levels, and hepatocellular proliferation and no change in serum cholesterol (Kocarek and Reddy, 1996; Cohen, 2010). No measure of HMG-CoA-reductase was conducted in the course of the fluopyram mechanistic work. Fluopyram was shown to increase serum cholesterol concentrations and mildly suppress *Cyp4a1* gene expression, both of which are opposite findings to those seen following exposure to known statins. These data demonstrate that fluopyram is not acting as a statin in rodents, and this MOA is unlikely to be responsible for fluopyram-induced cancers.

Receptor and non-receptor mediated liver cytotoxicity. The induction of liver damage following chemical exposure is a frequent finding in repeat-dose regulatory toxicology studies and can be evaluated by measuring liver specific enzymes. Biochemical markers such as alanine aminotransferase (ALT), alkaline phosphatase (ALP), and bilirubin are often used to evaluate hepatic damage. Liver injury is defined as a rise in either: 1) ALT > 3x upper limit of normal, 2) ALP > 2x upper limit of normal, or 3) total bilirubin level

> 2x upper limit of normal when associated with increased ALT or ALP (Holt and Ju, 2006).

Table 33. Clinical chemistry results for cholesterol (Chol), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and bilirubin from top dose group male and female Wistar rats exposed to fluopyram for 1, 3, 6, or 12 months.

Rat		Male		Female	
1 month (28 days)		Control	3200 ppm	Control	3200 ppm
	Chol. (mmol/l)	1.45	**2.64**	1.9	**3.5**
	ALT (IU/l)	37	32	32	30
	ALP (IU/l)	127	84	90	**54**
	Bilirubin (µmol/l)	1.0	1.4	1.5	1.0
3 months (90 days)		Control	3200 ppm	Control	3200 ppm
	Chol. (mmol/l)	1.87	2.95 (2.33)	1.88	**3.78 / 2.35**[r]
	ALT (IU/l)	41	36	29	30 / 34[r]
	ALP (IU/l)	74	57	47	31 / 43[r]
	Bilirubin (µmol/l)	2.0	1.3	2.6	1.7 / 2.5[r]
6 months		Control	750 ppm	Control	1500 ppm
	Chol. (mmol/l)	1.87	**2.14**	2.07	**2.72**
	ALT (IU/l)	43	40	42	44
	ALP (IU/l)	71	63	40	37
	Bilirubin (µmol/l)	4.2	4.2	3.9	4.0
12 months		Control	750 ppm	Control	1500 ppm
	Chol. (mmol/l)	2.01	2.0	1.98	**2.74**
	ALT (IU/l)	49	38	45	51
	ALP (IU/l)	65	57	32	29
	Bilirubin (µmol/l)	1.7	**1.1**	2.9	**1.6**

Bold represents statistically significant differences from the relevant controls. IU = International unit. [r] 1 month recovery.

These results show that the rat has no clinical chemical signature associated with hepatic injury, paralleling the lack of hepatic focal necrosis. The primary clinical chemistry finding in the rat was elevated cholesterol that was more severe in the female than male. The observation of increased cholesterol in rats is common following exposure to known Car/Pxr inducers such as phenobarbital (Thomas, 1984; Kiyosawa *et al.*, 2004).

Table 34. Clinical chemistry results for cholesterol (Chol), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and bilirubin in male and female C57BL/6J mice exposed to fluopyram for 28 or 90 days.

Mouse		Male				Female			
1 month (28 days)	Dose (ppm)	Control	–	150	1000	Control	–	150	1000
	Chol. (mmol/l)	1.69	–	**1.2**	**1.19**	1.31	–	**0.93**	**1.42**
	ALT (IU/l)	37	–	30	**133**	67	–	62	90
	ALP (IU/l)	130	–	128	148	192	–	191	170
	Bilirubin (µmol/l)	1.8	–	**0.9**	**0.8**	1.2	–	1.1	1.1
3 months (90 days)	Dose (ppm)	Control	30	150	1000	Control	30	150	1000
	Chol. (mmol/l)	1.35	**0.94**	**0.79**	**0.81**	1.22	1.08	**0.93**	1.33
	ALT (IU/l)	40	88	55	**122**	83	56	64	**96**
	ALP (IU/l)	96	98	96	**116**	157	153	148	144
	Bilirubin (µmol/l)	2.3	1.7	**1.3**	**1.3**	1.7	1.6	1.4	1.1

Bold represents statistically significant differences from the relevant controls. IU = International unit.

Significant elevations in ALT were seen in both male and female mice exposed to 1000 ppm fluopyram at 28 and 90 days and ALP in males at 90 days. This corresponds with increased hepatic focal necrosis seen in 20% and 80% of male mice at 3 and 14 days, respectively, when exposed to 2000 ppm fluopyram. Mice exposed to 1000 ppm fluopyram for 28 days saw 100% of males and 0% of females showing evidence of focal hepatocellular necrosis. Exposure to 750 ppm fluopyram for 28 days resulted in liver enlargement, hepatocellular hypertrophy, and mild focal hepatocellular necrosis in WT mice, but these were not seen in Pxr-Car-KO mice.

From these data, it appears that the mouse, male more so than female, shows evidence of hepatic damage as evidenced through elevated ALT values and the pathological finding of focal hepatocellular necrosis, which are indicative of liver cytotoxicity. However, these observations were only seen at doses greater than those eliciting thyroid tumors in the mouse.

Cytotoxic chemicals that directly affect the liver without enzyme induction produce hepatocellular necrosis within 48 hours followed by a regenerative hyperplastic response (Maronpot *et al.*, 2010). If the cycle of necrosis followed by compensatory proliferation continues, liver enlargement may ensue. The histological characteristics of cytotoxicity-

related compensatory hepatocellular proliferation include fluid accumulation, fatty change in hepatocytes, inflammatory cell infiltration, fibrosis, and possibly granuloma formation (Glaister 1986; Evans and Lake, 1998); none of which were seen in rodents exposed to fluopyram.

For fluopyram, focal hepatocellular necrosis may be a very minor occurrence in the mouse that is only seen at doses at or greater than 750 ppm; however, this cytotoxic MOA does not play a role in increased thyroid hormone clearance and proliferation as observed in the mouse thyroid. As liver tumors were not observed in the mouse and no indication of hepatic damage or cytotoxicity was seen in the rat, the MOA for fluopyram-induced cancers do not appear to be acting through a cytotoxic MOA in either the mouse or rat.

Apoptosis

As described previously, inhibition of apoptosis may be listed as a separate key event for a Car/Pxr-related MOA; however, apoptosis in the rodent may not be observed as a component of this effect because mouse hepatocytes do not enter apoptosis as readily as rat hepatocytes (Bursch *et al.*, 2005; Lake, 2009). Also, inhibition of apoptosis as a component of a Car/Pxr-related hepatocellular tumor MOA primarily pertains to the tumorigenic progression of pre-neoplastic altered hepatic foci rather than occurring in phenotypically normal hepatocytes (Schulte-Hermann *et al.*, 1989; 1990; Kolaja *et al.*, 1996b; Whysner *et al.*, 1996; Lake, 2009). In addition, standard regulatory studies do not typically provide quantitative data on the incidence of apoptosis in the liver or other tissues, and short-term mechanistic studies are not lengthy enough to induce altered hepatic foci for apoptosis evaluation.

Hepatocellular toxicity can occur not only secondary to apoptosis but also from an increase in necrosis. Either or both of these findings can result in regenerative proliferation and, if sustained, in the development of liver tumors. With respect to fluopyram, no characterization of increased apoptosis was undertaken, as this was seen as only an associative event that does not inform the MOA of Car/Pxr activation (Cohen, 2010).

Non-receptor mediated, increased cell proliferation. MOAs for hepatocellular carcinogens that induce non-receptor mediated increased cell proliferation include cytotoxicity, infection, metal overload, or increased apoptosis (Cohen, 2010). Cytotoxicity is unlikely to be a relevant MOA for fluopyram as outlined above, and none of the animals used within the carcinogenicity studies had infections. Fluopyram does not appear to result in hepatic accumulation of iron or copper based on histopathological findings in the liver for studies of multiple durations in either mice or rats and therefore is not a potential contributing MOA for the rodent liver or thyroid tumors.

Fluopyram did not increase apoptosis in any of the previously described toxicity studies, although no direct measure was undertaken beyond standard histopathological analysis. Rat liver transcript levels of growth arrest and DNA-damage-inducible 45 beta (*Gadd45b*), which is increased following stressful growth arrest conditions and treatment with DNA-damaging agents and associated with cell proliferation and apoptosis, was marginally increased 1.5- and 1.7-fold after 7 days exposure to 600 and 1500 ppm fluopyram, respectively. *Gadd45b* is increased by phenobarbital-like compounds (Yamamoto *et al.*, 2010), and any increased seen by fluopyram is most likely to the result of Car/Pxr activation.

Table 35. Summary Evaluation for Other Possible MOAs for liver tumor formation

	DNA Reactivity	**AhR or Pparα Activation**	**Cytotoxicity (3-90 Days)**	**Increased Apoptosis**	**Estrogens, Statins, Metals, Infectious**
Strength of Association	All test negative	No increase in relevant gene transcripts	No changes in relevant clinical chemistry parameters & no hepatic focal necrosis	No histopath evidence; however difficult to determine microscopically	No histopath evidence
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 present, - indicates attribute absent, +/- indicates equivocal.

Thyroid. The induction of chemical-specific thyroid tumors in rodents may be caused by the following mechanisms (Capen, 1997; Hurley *et al.*, 1998): 1) DNA reactivity; 2) inhibition of the active transport of inorganic iodide into the follicular cell (iodide pump); 3) inhibition of thyroid peroxidase that converts inorganic iodide into organic iodide and couples iodinated tyrosyl moieties into thyroid hormone; 4) damage to follicular cells; 5) inhibition of thyroid hormone release into the blood; 6) inhibition of the conversion of T_4 to T_3 by 5'-monodeiodinase at various sites in the body and 7) enhancement of the metabolism and excretion of thyroid hormone by the liver, largely through the action of UGT.

Concerning the mechanism 1, as previously stated, based on *in vitro* and *in vivo* genotoxicity studies fluopyram is not a genotoxic chemical.

Mechanisms 2–5 are the result of direct thyroid gland effects, whereas 6 and 7 are peripheral to the thyroid gland. In examining these mechanisms in light of the fluopyram data, it is unlikely that mechanism #2 (inhibition of the active transport of inorganic iodide into the follicular cell), #3 (inhibition of thyroid peroxidase that converts inorganic iodide into organic iodide and couples iodinated tyrosyl moieties into thyroid hormone), or #5 (inhibition of thyroid hormone release into the blood) played a role in mouse thyroid tumor formation. Indeed, the evidence of an increased T4 clearance does not favor a direct effect of fluopyram on thyroid hormone biosynthesis and release to explain the decreased plasma T_4 levels induced by fluopyram. Moreover, mechanistic studies using hog thyroid microsomes showed fluopyram did not affect thyroid peroxidase (Bayer HealthCare report AT04481).

Microscopic examination of the thyroid gland in the mouse and rat never showed overt cytotoxicity, only hyperplasia/adenoma in the mouse following chronic treatment and hypertrophy and hyperplasia in the rat; thus, mechanism #4 (damage to follicular cells) is unlikely. As for the effects occurring outside the thyroid, mechanism #6 (inhibition of the conversion of T_4 to T_3 by 5'-monodeiodinase at various sites in the body) is unlikely as stated above; serum levels of T_3 were not changed in either the mouse or rat exposed to fluopyram. This indicates that mechanism #7 (enhancement of the metabolism and excretion of thyroid hormone by the liver, largely through induction of UGT enzymes) is the most likely mechanism, as fluopyram increased the activity UGTs involved in the clearance of T_4.

More importantly, the pivotal Pxr-Car-KO study provided compelling evidence to demonstrate that fluopyram is a Car-Pxr inducer and is not a direct thyroid toxicant. In this study, no induction of UGT-T4, no induction of *Tsh β* transcripts and no increased thyroid follicular cell proliferation was observed in KO mice following fluopyram treatment whereas all these effects were observed in wild type mice. These data demonstrate that the interaction of fluopyram with hepatic Car-Pxr nuclear receptors is obligatory to induce hepatic Phase II and pituitary *Tsh β* transcript and ultimately the

pre-neoplastic thyroid effects. The absence of thyroid related findings in KO mice would be anticipated in the case of an indirect thyroid toxicant acting via Car-Pxr activation. (Table 36).

Moreover, the findings in this KO mice study are inconsistent with a direct interference with thyroid hormone biosynthesis. Indeed, if fluopyram was directly interfering with thyroid hormone biosynthesis an increase in *Tsh b* transcripts and an increased thyroid follicular cell proliferation would be expected in both wild type and KO mice (Table 36). In addition a direct acting chemical would have also produced thyroid effects not only in rodents but also in the dog; this was not the case for fluopyram.

Table 36: Anticipated effects induced by direct thyroid toxicant and Car-Pxr activator in wild-type and Pxr-Car-KO mice.

	Car-Pxr activator (indirect)		Direct thyroid toxicant	
	Wild type	Pxr-Car-KO	Wild type	Pxr-Car-KO
Ugt-T4	↑	No change	No change	No change
T4 clearance	↑	No change	No change	No change
Plasma T4	↓	No change	↓	↓
Tsh β	↑	No change	↑	↑
TFC proliferation	↑	No change	↑	↑

Overall, from all the possible MOA that lead to thyroid hyperplasia and tumors, the most compelling MOA is the one involving hepatic Car-Pxr nuclear receptor activation.

6. Uncertainties, Inconsistencies, and Data Gaps.

Gender: ***Only female rats developed liver tumors.*** MOA investigations primarily focused on female rats and male mice, as fluopyram-induced tumors were found in these specific genders. In a study examining Wistar rats exposed to 500 ppm phenobarbital in drinking water for 152 weeks, 32% of females and 59% of males developed liver tumors (Rossi *et al.*, 1977). This was the same strain of rat used in the carcinogenicity studies for fluopyram; however, liver tumors were observed only in high-dose females that

received a higher dietary level of fluopyram compared to males, *i.e.,* 1500 ppm versus 750/375 ppm. As detailed in the fluopyram liver tumor MOA section, in the rat chronic study at 12 months, the incidence of altered hepatic foci were much higher in the male than female (50% and 30%, respectively). However, after 21 months on study at 750 ppm, adverse clinical signs and mortality in the male rat required lowering this level to 375 ppm. This resulted in an equivalent incidence of altered hepatic foci in males at 12 and 24 months (50% versus 48%), whereas the female rat (maintained at 1500 ppm fluopyram), saw an increase from 30 to 81% at 12 and 24 months, respectively. If the male rat continued the carcinogenicity study at 750 ppm, there would have been a compromise in the ability of that dose group to survive, which could have possibly compromised the statistical analysis of that group. However, it is highly probable that if some male rats did survive an exposure to 750 ppm fluopyram for 24 months, liver tumors would have developed as seen in the female rat.

Gender and species specificity for tumor formation: *Why did only male mice developed thyroid tumors?* Within the fluopyram data for the mouse, all of the thyroid mechanistic studies were conducted in the male mouse. This was justified by reducing animal use and examining the most sensitive gender. The increase in treatment-related thyroid tumors in the male mouse compared with the female mouse is consistent with the higher level of liver induction observed in male at the histopathological examination in the mouse chronic study (SA 05094). In this study, a higher incidence and severity of hepatocellular hypertrophy was observed in males compared to females at both 150 and 750 ppm, in addition, multinucleated hepatocytes and inclusion bodies were only observed in the males. Supporting this approach, a review of pesticide-induced thyroid tumors found the male rat to be more sensitive to thyroid carcinogens than females (Hurley *et al.*, 1998). Furthermore, in a U.S. Food and Drug Administration report on drugs that caused thyroid tumors, a total of 18 were identified with eight occurring only in males, one only in females, and the remainder in both males and females (Contrera *et al.*, 1997). Although these observations are in rats, it may lend some understanding of the observed gender bias. For example, male rats tend to have higher levels of TSH following exposure to thyroid carcinogens (Hill *et al.*, 1998).

Fluopyram induced thyroid tumors in the mouse, not in the rat. This is opposite to the majority of findings in the literature (reviewed in Hurley *et al.*, 1998) that indicated that of the 21 pesticides identified to have induced thyroid tumors, all were in the rat with only two pesticides inducing thyroid tumors in the mouse. Thus, no pesticide has ever induced thyroid tumors in the mouse without also doing so in the rat. All rat thyroid pesticide carcinogens induced tumors in the male (100%) but only 76% in female rats. It is clear when examining the totality of the fluopyram data that the thyroid of the rat was adversely impacted, as was the liver in the mouse. However, over the duration of the chronic studies, male and female mice were exposed to higher levels of fluopyram on a mg/kg/day basis compared to rats (105 and 129 mg/kg/day in the male and female mouse, respectively, compared with 29 and 89 mg/kg/day in the male and female rat, respectively). Female rats, which came considerably closer than the male rat to the mg/kg/day exposure of the mice, developed both thyroid follicular cell hypertrophy and hyperplasia, whereas male rats developed only thyroid hypertrophy. Due to the toxicity profile observed in the 90-day rat study (SA 04048), where kidney nephropathy was observed in males at ≥ 200 ppm, at the outset of the chronic study (SA 04312), the male high dose group received 750 ppm fluopyram in the diet, which was subsequently reduced to 375 ppm after 84 weeks of treatment due to the high mortality rate observed in this group. Female rats allocated to the high dose group received 1500 ppm fluopyram on the diet throughout the study duration. Over the course of the chronic, the high dose males received 29 mg/kg/day of fluopyram compared with 89 mg/kg/day in the corresponding female dose group. It is therefore likely that thyroid adenomas would have been observed in female rats if they had been exposed to >89 mg/kg/day.

Lack of reliable hormone measurement data for T_4 and TSH in the plasma. During the course of this mechanistic program it was technically challenging to obtain reliable measurements for plasma levels of T_4 and TSH. Although a dose related decrease in plasma T_4 levels due to fluopyram treatment was demonstrated in two oral gavage studies using high dose levels (100 and 300 mg/kg/day ie ~750 and ~2000 ppm respectively [SA 10430, SA 10241]) , a clear dose response relationship could not be established when treatment was via the diet at doses ≤750 ppm. Several factors could have

contributed to this shortcoming such as the fact that the hormone measurements were taken following dietary administration. With respect to plasma TSH measurements, it was difficult to identify alterations in TSH concentrations during the entire mechanistic program. This is likely due to the fact that all thyroid-related effects induced by fluopyram were weak. Consequently, we used *Tsh b* transcripts as a surrogate marker of plasma TSH and consider the increase in this biomarker as evidence for an increase in TSH.

Overall, in a weight of evidence approach, even if clear plasma hormone data were difficult to obtain, the effects induced by fluopyram in the standard and mechanistic studies excluded the potential of fluopyram to be a direct thyroid toxicant and demonstrate that fluopyram is a Car-Pxr activator.

Lack of liver tumors in the C57BL/6J mouse. Although in many of the mechanistic and repeat-dose studies on fluopyram, the mouse liver presented as more severely impacted than the rat, the mouse did not develop liver tumors. This is primarily a result of characteristics of the C57BL/6J mice making it less susceptible to liver tumor induction when exposed to compounds such as phenobarbital (Yamamoto *et al.*, 2004). It is unknown why the C57BL/6J mouse develops 90% fewer liver tumors than the susceptible C3H/He mouse strain following exposure to phenobarbital (Diwan *et al.*, 1986), but it is thought to be related to differences in the degree of hepatocellular proliferation and growth regulation of initiated hepatocytes leading or not to tumor promotion*et al.*,. This strain specificity is likely to be genetically controlled by multiples genes termed Hcs (hepatocarcinogen sensitivity genes) (Drinkwater and Ginsler 1986; Goldsworthy and Fransson-Steen 2002; Bursch *et al*., 2004).

Although the mouse is generally more susceptible to liver tumorigenesis following exposure to phenobarbital, (Rossi *et al.*, 1977; Butler, 1978; Whysner *et al.*, 1996; Hagiwara *et al.*, 1999), rats exposed to other known inducers of Car, such as metofluthrin or pyrethrins, develop liver tumors, whereas none are seen in the mouse (Deguchi *et al.*, 2009; Yamada *et al.*, 2009; Osimitz and Lake, 2009). Furthermore, microsomal enzyme inducers such as phenobarbital, pregnenolone-16α-carbonitrile, 3-methylcholanthrene,

and Aroclor 1254 were found to induce UGT-T4 activity and increase serum TSH and thyroid follicular cell proliferation via a mechanism of altered thyroid hormone homeostasis in mice (Bookstaff *et al.*, 1996; Hood *et al.*, 2003) and rats (Hood *et al.*, 1999; Liu *et al.*, 1995). Thus, both rodent species should be considered susceptible to liver and thyroid tumor formation when exposed to a Car/Pxr inducer.

Mechanistic data focused only on female rat liver and male mouse thyroid. As stated at the beginning of this assessment, tumors were seen in the female rat liver and male mouse thyroid. To reduce the number of animals, mechanistic studies focused on the more sensitive gender and species. As such, there is little mechanistic information on the female mouse and male rat. Because of the lack of data in these areas, a complete understanding of the differences between gender and strain is more difficult to obtain and adds to the uncertainty of the possible effects within these less-examined groups. However, as the mechanistic investigations were conducted within the group showing the induction of tumors, it is reasonable to conservatively assume that effects occurring within the less sensitive group are covered by the data generated from the more sensitive (tumorigenic) group.

7. Assessment of Postulated Mode of Action

The key events for fluopyram-induced rat liver and mouse thyroid tumors were successfully identified, with the majority of key events shown to be reversible, and all had a dose- and temporal-response. Moreover, Pxr-Car-KO mice were refractory to the Car/Pxr-mediated hepatic and thyroid effects seen in WT mice. Overall, the data informing the MOA of fluopyram provide a strong and consistent association with the MOA known to induce liver and thyroid tumors in rodents following exposure to Car and Pxr inducers. The data presented within this framework document are considered, with a high degree of confidence, adequate to explain the development of fluopyram-induced tumors in female rats and thyroid tumors in male mice following chronic dietary exposure to fluopyram.

8. Applying the International Programme on Chemical Safety Human Relevance Framework to Fluopyram.

Question 1. Is the weight of evidence sufficient to establish the MOA in animals?

Yes; the MOA for fluopyram-induced mouse thyroid and rat liver tumors is compatible with that described for other Car/Pxr-induced rodent thyroid and liver tumors (Whysner *et al.*, 1996; Holsapple *et al.*, 2006; Lake, 2009; Cohen, 2010; Hurley *et al.*, 1998; Dellarco *et al.*, 2006). The relevant molecular and pathological endpoints for fluopyram-induced liver effects in rats and mice are supported by repeat-dose rat and mouse studies, and there is good correlation for the dose response between the MOA data and rat liver tumor occurrence.

Key event #1 for the fluopyram liver and thyroid tumor MOA is defined as activation of the Car/Pxr nuclear receptors. This was measured by induction of Cyp2b and Cyp3a transcript and enzyme activity starting at 30 ppm (enzyme) in mouse and 30 ppm (transcript) and 600 ppm (enzyme), respectively, in the rat. Supportive, associative data to key event #1 included increased liver weight and hepatocellular hypertrophy. Liver key event #2 was an increase in global hepatocellular proliferation, and key event #3 was

altered hepatic foci, which occurred at ≥ 150 ppm fluopyram respectively in rats. All of the liver key events occurred at ≥ 150 ppm, and liver tumors were observed in female rats at 1500 ppm following chronic exposure.

Thyroid key event #1 was the induction of liver Phase 1 metabolic enzymes followed by key event #2, the induction of UGTs and consequent increased T_4 clearance and reduced plasma T_4 levels. Additionally, thyroid key events #3 (increased TSH), #4 (increased thyroid follicular cell proliferation) and #5 (increased thyroid follicular cell hyperplasia) were recorded. Dose-related effects for key events 2, 4 and 5 were observed from 150 ppm, whereas for Key event #3, measured using a surrogate biomarker dose-related effects were only observed from 600 ppm. This difference in dose response concordance may be explained by a lack of sensitivity of the surrogate biomarker used for TSH measurement.

Finally, key events for the liver and thyroid demonstrated dependence on rodent Car/Pxr involvement, as Pxr-Car-KO animals did not respond similarly to WT mice. When all of the mechanistic and standard studies for fluopyram are analyzed, the key events show clear dose-response alterations that are consistent with a Car/Pxr-mediated MOA. In addition, other possible MOAs were examined and determined to be unlikely based on analysis of the relevant data.

Question 2. Can human relevance of the MOA be reasonably excluded based on fundamental qualitative differences in key events between experimental animals and humans?

Yes; activation of the Car and Pxr nuclear receptors and subsequent induction of CYP enzyme activity in the liver following exposure to Car and Pxr inducers (such as phenobarbital) is a well-understood MOA for rodent liver and thyroid tumors (Whysner *et al.,* 1996; Cohen, 2010; Dellarco *et al.*, 2006). Phenobarbital is used to treat epilepsy and anxiety in humans. After decades of use it has been deemed safe for humans, with no increase in associated tumors. However, rodents exposed to high levels of phenobarbital for the majority of their lives develop both liver and thyroid tumors. So why is there a difference in tumor response between humans and rodents? The key events identified in Car/Pxr-associated rodent tumor formation are: 1) activation of the

Car and Pxr receptors and induction of detoxification enzymes; 2) increased liver proliferation; and 3) pre-neoplastic foci, which can ultimately result in the generation of a tumor. In humans, only the first step of this progression occurs following exposure to phenobarbital. Because liver proliferation is not induced, the following steps that lead to tumors do not occur.

Activation of rodent Car and Pxr produces a cascade of alterations in gene transcription that leads to increased hepatocellular proliferation, a critical event in the development of liver tumors (Whysner *et al.,* 1996; Cohen, 2010; Capen, 1997). On the other hand, phenobarbital in humans results in activation of Car and Pxr, leading to the induction of CYP enzymes but there is no evidence of increased hepatocellular or thyroid follicular cell proliferation in humans or primary human hepatocytes *in vitro* (Lake, 2009). This finding was reinforced by demonstrating that human primary hepatocytes were refractory to the hepatocellular proliferative effect of fluopyram, whereas rat primary hepatocytes showed a significant increase in proliferation (Figure 4). Extensive epidemiologic studies in humans exposed to levels of phenobarbital comparable to those in rodent bioassays did not find an increased risk of cancer, and phenobarbital is not considered to be a cancer risk to humans (Whysner *et al.,* 1996; Lamminpaa *et al.*, 2002; Holsapple *et al.,* 2006). On this basis, the rodent liver tumors associated with administration of fluopyram would not pose a cancer hazard to humans.

The key events in Car/Pxr-mediated liver tumorigenesis include activation of these receptors, induction of Cyp2b and Cyp3a gene and enzymes, increased hepatocellular proliferation with subsequent induction of proliferative lesions in the liver including foci, adenomas, and carcinomas. The key events in Car/Pxr-mediated thyroid tumorigenesis, in addition to including activation of these receptors and induction of Cyp2b and Cyp3a gene and enzymes, also requires the induction of Phase II enzymes (such as UGTs) that clear thyroid hormones from the system while aiding in the removal of fluopyram. These result in lower circulating T_4 levels, increased TSH, and consequent increased thyroid follicular cell proliferation, with subsequent induction of proliferative lesions in the thyroid, including adenomas and carcinomas.

Thyroxine-binding globulin is the predominant plasma protein in humans and non-human primates (but not rodents), which binds and transports thyroid hormone in the blood (Hill *et al.*, 1989). This protein has binding affinities three and five orders of magnitude greater than the other two thyroxine-binding proteins, albumin and pre-albumin, respectively. The lack of thyroxine-binding globulin in the adult rat is another important difference. Additional differences are the half-life of T_4 (12 hours in the rat versus 5–9 days in humans) and in the serum level of TSH, which is 25 or more times higher in the rodent than in humans (Dohler *et al.*, 1979; McClain 1992). The rat also exhibits enhanced thyroid hormone elimination with less efficient enterohepatic recirculation compared to humans. The histology of the non-stimulated rodent thyroid is similar to that of the stimulated human gland, with small follicles lined by tall follicular cells. Thus, the physiological parameters and the histological appearance indicate that the rodent thyroid gland is more active and operates at a higher level with respect to thyroid hormone turnover as compared to the human gland (Dellarco *et al.*, 2006).

A concordance analysis of the key events for the Car-Pxr-mediated liver (Table 37) and thyroid (Table 38) MOA are presented below.

Table 37. Concordance of Key Events for a Car/Pxr mediated liver tumor MOA in Rodents and Humans.

Key Event	Evidence in Rodents	Evidence in Humans
Activation of Car/Pxr	Yes.	Yes.
CYP Enzyme Induction Liver Hypertrophy	Yes; unclear if critical step or indicator of activity secondary to Car/Pxr activation.	Yes; different enzymes induced compared to rodents.
Hepatocellular Proliferation	Yes.	No evidence of increased cell proliferation in the human liver (both *in vitro* and *in vivo* data).
Selective Clonal Expansion (Foci)	Yes.	No; none reported.
Occurrence of Hepatocellular Tumors	Yes.	No; based on PB epidemiological data.

Table 38. Concordance of Key Events for a Car/Pxr mediated thyroid tumor MOA in Rodents and Humans.

Key Event	Evidence in Rodents	Evidence in Humans
Activation of Car/Pxr	Yes.	Yes.
CYP Enzyme Induction	Yes.	Yes; however slightly different enzymes induced compared to rodents.
Increased T_4 clearance	Yes.	Yes/No; when seen, only very mild reduction when treated with anti-convulsants such as PB*.
Increased TSH	Yes.	No; not seen following exposure to anti-convulsants*.
Increased thyroid cell proliferation	Yes.	No; none reported.
Occurrence of Thyroid Tumors	Yes.	No; based on epidemiological data.

* Benedetti *et al.* (2005).

In a study examining the proliferative response in rat and human primary hepatocytes exposed to fluopyram, rat cells showed a dose-response increase in proliferation, whereas human cells did not (Figure 2). Phenobarbital and epidermal growth factor were used as positive controls, with phenobarbital reciprocating the fluopyram results, and epidermal growth factor producing a strong proliferative response in both rat and human cells. This simple experiment clearly demonstrates the difference between the rodent and human hepatocyte proliferative response when exposed to compounds that induce Car and Pxr.

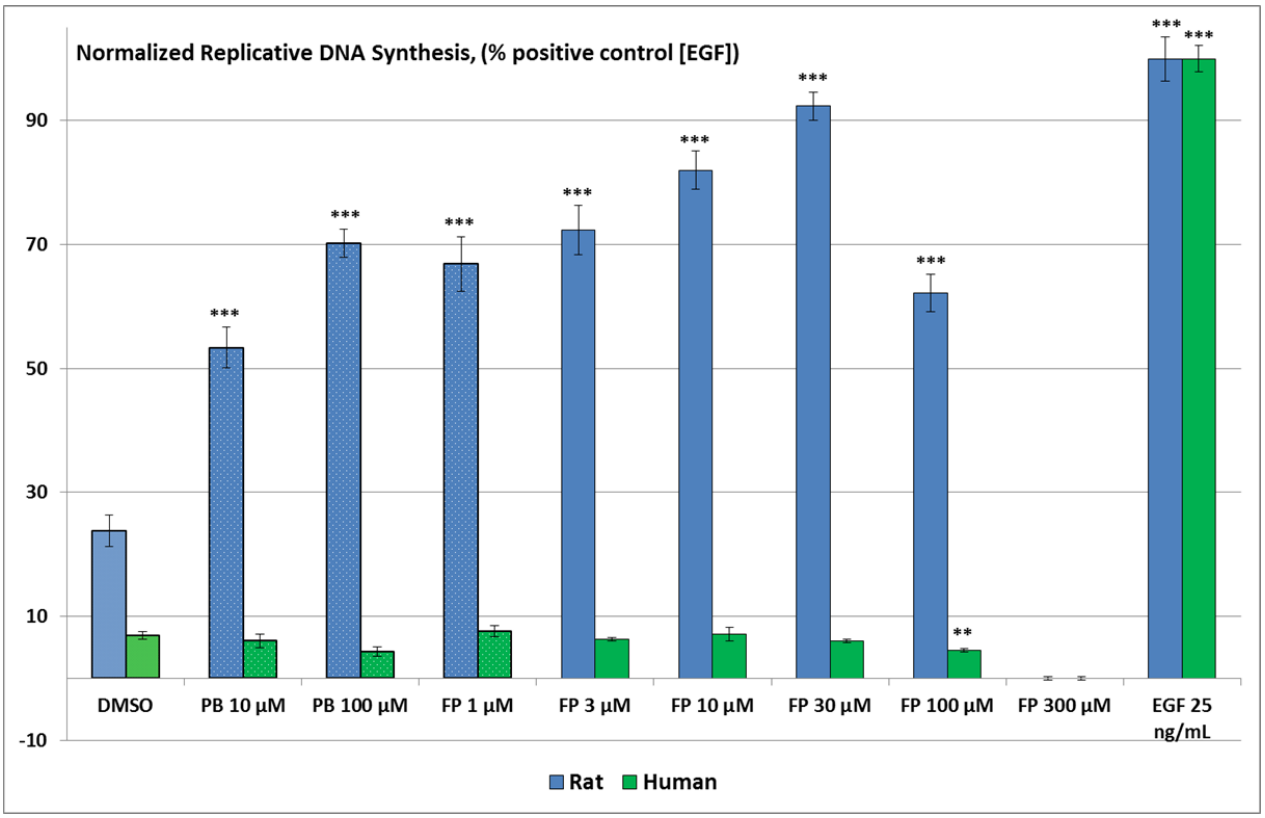


Figure 2. Proliferative response in rat and human primary hepatocytes exposed to fluopyram (FP) given as normalized replicative DNA synthesis compared to epidermal growth factor (EGF). Phenobarbital (PB) and EGF were used as positive controls. Fluopyram was slightly cytotoxic at 100 µM with 100% mortality at 300 µM. Rat data is from CXR study CXR1242 and the human data from CXR study CXR1241. $p<0.05$; ** $p<0.01$; ***$p<0.001$.

Question 3. Can human relevance of the MOA be reasonably excluded based on quantitative differences in either kinetic or dynamic factors between experimental animals and humans?

As human relevance of the experimental animal MOA can be reasonably excluded on the basis of qualitative differences in key events (Question 2), a quantitative assessment of kinetic or dynamic factors is not necessary.

Conclusions

Statement of confidence in the evaluation. The data composing the MOA for fluopyram were applied to the criteria proposed by Bradford and Hill and those of the Human Relevance Framework to the rat liver and mouse thyroid tumors following the guidelines established for this process (Sonich-Mullin *et al.*, 2001; Cohen *et al.*, 2003; Meek *et al.*, 2003; USEPA, 2005; Holsapple *et al.*, 2006; Boobis *et al.*, 2007). The extensive toxicological database for fluopyram, including several focused MOA studies in both mice and rats, as well as studies using genetically engineered KO mice and primary rat and human hepatocytes consist of high-quality studies that provide the necessary data to determine the MOA for fluopyram-induced rodent tumors.

Key Event #1 for both thyroid and liver non- neoplastic and neoplastic lesions is defined as activation of the liver Car/Pxr nuclear receptors, which is measured by the induction of Cyp2b/Car and Cyp3a/Pxr-associated enzyme activities. Key Event #1 was further confirmed by the absence of Cyp2b/Cyp3a enzyme induction in Pxr/Car KO mice exposed to fluopyram. Supportive, associative events to key event #1 include increased liver weight and microscopic hepatocellular hypertrophy.

Liver Key event #2 is an increase in hepatocellular proliferation and was identified in female rats. Liver Key Event #3 was an increase in altered hepatic foci that progressed to tumors only in the female rat.

Thyroid Key Event #2 was induction of UGT enzymes specifically involved in the clearance of thyroid hormones leading to a reduction in plasma T_4, which was apparent in the male mouse. Key Event #3 was increased TSH secretion, which led to thyroid Key Event #4, increased thyroid follicular cell proliferation. Importantly, the Pxr-Car-KO

mice showed no increase in UGT enzyme activity, no increase in TSH secretion and no increase in thyroid follicular cell proliferation, demonstrating the obligatory role of these nuclear receptors to initiate the thyroid-altering cascade observed following exposure to fluopyram.

The key events for fluopyram show clear threshold dose-response alterations and provide informative, temporal-specific characterization of fluopyram-induced liver and thyroid effects. These key events are consistent with a Car/Pxr-mediated MOA. Other possible MOAs for receptor- and non-receptor-mediated tumorigenesis, as described by Cohen (2010) for the liver and Capen (1997) and Hurley *et al.* (1998) for the thyroid, have been evaluated with respect to fluopyram and dismissed because of a lack of plausibility and coherence.

Implications for risk assessment. There is convincing evidence that the MOA for fluopyram-induced thyroid and liver effects in the mouse and rat, respectively, do not occur below a threshold dose level. Specifically, the two key events of the MOA prior to tumor formation in the rat liver, increased cell proliferation and altered hepatic foci, occurred at ≥ 150 ppm, with tumors at 1500 ppm. In the mouse, the two key events of the MOA prior to tumor formation in the thyroid, increased thyroid follicular cell proliferation and hyperplasia, started from 150 ppm, with tumors at 750 ppm. Consequently fluopyram should be considered as a threshold carcinogen and on this basis, a threshold approach rather than a quantitative (Q*) assessment for risk assessment should be applied to the mouse thyroid and rat liver tumors associated with fluopyram
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 indicate that the rat liver and mouse thyroid tumors associated with fluopyram exposure is unlikely to occur in humans.

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Appendix (in order by earliest year)

[redacted] (2004); AE C656948 - Exploratory 28-day toxicity study in the rat by dietary administration; Bayer S.A.S., Bayer CropScience; Report No.: SA 03332; Document No.: M-085510-01.

Summary: The potential systemic toxicity of AE C656948, a fungicide of the pyramide family (batch number FLH 999: a pale yellow powder, 98.6% (w/w) purity), was administered continuously via the diet to groups of Wistar rats (5/sex/group) for 28 days at concentrations of 50, 400 and 3200 ppm, corresponding to 4.0 and 4.6, 31.0 and 36.1, 254 and 263 mg/kg/day in males and females, respectively. A similar constituted group received untreated diet and acted as a control group. Animals were observed daily for mortality and clinical signs. A detailed physical examination was performed weekly. Body weight and food consumption were recorded weekly. Blood samples were taken before final necropsy for hematology and clinical chemistry determinations. At study termination, all animals were necropsied, selected organs weighed and a range of tissues were taken, fixed and examined microscopically. The remaining portions of the liver were homogenized for microsomal preparations in order to determine cytochrome P-450 isoenzyme profile. AE C656948 dietary administration to male and female Wistar rats at 3200, 400 or 50 ppm for 28 days induced no mortalities or clinical signs. At 3200 ppm, mean body weight gain per day was reduced by between 12 to 29% during study Weeks 1 and 3 in both sexes and also during study Week 4 in females, when compared to the control values. The overall mean cumulated body weight gain was comparable to the control value in males, but reduced by 14% in females. Mean food consumption was reduced throughout the study by between 4 to 10% in females. At the hematology evaluation, mean platelet count was increased by 30% and mean prothrombin time by 34% in males. Clinical chemistry assessment revealed higher mean total cholesterol and triglycerides concentrations together with slightly lower mean aspartate aminotransferase and alkaline phosphatase activities in both sexes. In addition, a tendency towards lower glucose concentrations was seen in males and higher mean total protein and albumin concentrations were observed in females. At necropsy, mean absolute and relative liver weights were increased by 54 to 74% in both sexes. In addition in males, mean absolute and relative kidney weight was increased by 18 to 21% and mean absolute and relative thyroid gland weights were increased by 43 to 46%. At the macroscopic examination, enlarged and dark livers were observed in almost all males and females, together with pale kidneys in all

males. Microscopic examination revealed treatment-related changes in the liver, thyroid gland and kidney in both sexes, and in the pituitary gland in males only. Minimal to moderate hypertrophy of centrilobular hepatocytes was present in all animals. Minimal to slight diffuse hypertrophy of follicular cells was seen in the thyroid gland in 3/5 males and 2/5 females, together with depletion of colloid in 2/5 males. Minimal diffuse hypertrophy of basophils was noted in the pituitary gland in 3/5 males. Hyaline droplet nephropathy related to the accumulation of $\alpha_{2\mu}$-globulin in the proximal tubules was noted in all males and in 1/5 females. However, this finding was considered not toxicologically relevant to man as $\alpha_{2\mu}$-globulin is present only in trace amount in humans. At the hepatic evaluation, AE C656948 was found to be a moderate phenobarbital-like cytochrome P-450 inducer, causing a slight increase in total cytochrome P-450 and a marked induction in BROD and PROD activities in both sexes.

At 400 ppm, treatment-related changes were mainly noted in males. At necropsy, mean absolute and relative liver weights were slightly increased by between 7 to 21 % in both sexes. In addition, mean absolute and relative kidney weights were increased by 15 to 20% in males. At the macroscopic examination, pale kidneys were observed in 3/5 males, a dark liver was observed in 1/5 males and an enlarged liver in 1/5 females. Microscopic examination revealed treatment-related changes in the liver in both sexes and in the kidney in males only. Minimal hypertrophy of centrilobular hepatocytes was noted in all males and in 1/5 females. Hyaline droplet nephropathy was observed in all males, but this finding was considered not toxicologically relevant to man. At the hepatic evaluation, AE C656948 induced a slight increase in total cytochrome P-450 and a moderate increase in BROD and PROD activities in both sexes. At 50 ppm, examination of the kidneys revealed a higher incidence of hyaline droplets containing $\alpha_{2\mu}$-globulin in the proximal tubules at a minimal severity in males, but this finding was considered not toxicologically relevant to man. No treatment-related effects were observed in females. Hence, this dose level represented the No Observed Adverse Effect Level (N.O.A.E.L.) of AE C656948 in males and the No Observed Effect level (N.O.E.L.) in females (equating to 4.0 and 4.6 mg/kg body weight/day in males and females, respectively).

[redacted]. (2004); AE C656948 - Preliminary 28-day toxicity study in the mouse by dietary administration; Bayer S.A.S., Bayer CropScience; Report No.: SA 04013; Document No.: M-088486-01.

Summary: AE C656948, a fungicide of the pyramide family (batch number FLH 1046: a beige powder, 99.4% purity) was administered continuously via the diet to groups of C57BL/6 J mice (5/sex/group) for 28 days at concentrations of 150, 1000 and 5000 ppm, corresponding to 24.7, 31.1, and 162 in males and 197, 747, and 954 mg/kg/day in females, respectively. A similarly constituted group received untreated diet and acted as a control group. Animals were observed daily for mortality and clinical signs. Detailed physical examinations were performed weekly. Body weight and food consumption were recorded once weekly. In addition, animals were weighed at final sacrifice. Selected clinical chemistry parameters were determined at the end of the study. All animals were subjected to necropsy, selected organs weighed and a range of tissues were fixed and examined microscopically.

At 5000 ppm

All males and 3/5 females were sacrificed for humane reasons between study Days 17 and 27. Clinical signs in these decedent animals comprised of reduced motor activity, hunched posture, piloerection, wasted appearance and cold to touch in both sexes together with abnormal respiration in 3/5 males and distended abdomen in 2/3 females. These clinical signs were noted mainly on the day of sacrifice or for a few days prior to sacrifice. A mean body weight loss of 1.1 g and a 28% decrease in mean food consumption were observed during study Week 2 prior to the early sacrifices in males, whilst a mean body weight loss of 0.9 g and a 11% decrease in mean food consumption were noted during study Week 3 prior to the three early sacrifices in females. At necropsy, a pale pancreas was observed macroscopically in all males and in 2/3 females. Rounded borders in the liver were observed in 3/5 males and 1/3 females. Dark livers were observed in 4/5 males and in all females, and enlarged livers were observed in 1/5 males and 2/3 females. The size of thymus was clearly reduced in 4/5 males and 1/3 females, and distended abdomen was noted in 3/5 males. Red liquid was observed in the thoracic cavity in all males. At the microscopic examination, hypertrophy, vacuolation and degeneration/necrosis of the zona fasciculata were seen in the adrenal glands in all animals, together with perivascular and intra-alveolar hemorrhage and degeneration/inflammation of pulmonary veins in the lungs and erythroid extramedullary hematopoiesis in the spleen. Focal hemorrhage was seen in the thyroid gland in 3/5 males and decreased cellularity of

the cortex and focal hemorrhage were seen in the thymus in all animals where examination was possible. In the liver, hypertrophy of hepatocytes, hepatocellular eosinophilia, bile duct/oval cell hyperplasia, focal necrosis and single cell hepatocellular necrosis were seen in all animals, and centrilobular degeneration/necrosis in 1/5 males. The increase in the incidence and severity of extramedullary hematopoiesis in the spleen was considered to most likely represent a reactive response to the intrathoracic hemorrhaging noted in moribund animals. The lesions in the adrenal glands and decreased cellularity of the thymic cortex are consistent with stress as a non-specific reaction rather than a direct effect of treatment. In the two surviving females at 5000 ppm, a distended abdomen was noted between study Days 8 and 10 in one animal. Clinical chemistry assessment revealed high total cholesterol (+118%) and total protein (+16%) concentrations, as well as high alanine aminotransferase activities (+384%) for both females. At necropsy, mean absolute and relative liver weights were increased by between 132 and 147%, when compared to the control values. Macroscopic examination revealed enlarged livers in both females and dark livers in 1/2 females. At the microscopic examination, hypertrophy of the zona fasciculata was seen in the adrenal glands in both animals. In the liver, hypertrophy of centrilobular hepatocytes, focal necrosis, hepatocellular eosinophilia and bile duct/oval cell hyperplasia were noted in both females, together with a single hepatocellular necrosis in 1/2 females.

At 1000 ppm

There were no mortalities, nor clinical signs. A slight decrease in the daily mean body weight gain was noted during study Week 2 in males (0.03 g/day vs. 0.10 g/day in the control group). At the clinical chemistry assessment, mean alanine aminotransferase activity was increased by 259% in males and mean albumin concentration was decreased by 12% in females. Mean total bilirubin and albumin concentrations were decreased in males by 56 and 10%, respectively, compared to controls. These changes were considered to be treatment-related, but most likely not toxicologically relevant as the individual values were within historical control ranges. At necropsy, mean absolute and relative liver weights were increased by between 27 and 49% in both sexes, when compared to the control values. Macroscopic examination revealed enlarged livers in all males and in 4/5 females, and dark livers were observed in 3/5 males and in 2/5 females. At the microscopic examination, hypertrophy of the zona fasciculata was seen in the adrenal glands in 3/5 females. In the liver, hypertrophy of centrilobular hepatocytes was observed in all animals in both sexes, single

hepatocellular necrosis was seen in all males, focal necrosis was noted in 3/5 males and 2/5 females, and hepatocellular eosinophilia and bile duct/oval cell hyperplasia were seen in 1/5 females.

At 150 ppm

Treatment-related findings comprised of a 50% decrease in mean total bilirubin and a 5% decrease in albumin concentrations in males. These changes were considered to be treatment-related, but likely not toxicologically relevant as the individual values were within historical control ranges. At necropsy, mean absolute and relative liver weights were increased by between 15 and 21% in both sexes, when compared to the control values. Microscopic examination revealed minimal to slight hypertrophy of centrilobular hepatocytes in the liver in all males and 2/5 females. This finding was consistent with increased liver weights at necropsy and, in isolation, was considered to be associated with adaptive changes rather than evidence of toxicity.

In conclusion, a dose level of 5000 ppm clearly exceeded the Maximum Tolerated Dose (M.T.D.) due to the overt toxicity noted, whilst a dose level of 150 ppm represented the No Observed Adverse Effect Level (N.O.A.E.L.) of AE C656948 in both sexes (equating to 24.7 mg/kg body weight/day in males and 31.1 mg/kg body weight/day in females).

[redacted] (2005); AE C656948 – 90-day toxicity study in the rat by dietary administration; Bayer S.A.S., Bayer CropScience; Report No.: SA 04048; Document No.: M-250946-01.

Summary: This study was conducted according to the O.E.C.D. guideline 408 (1998), E.E.C. directive 2001/59/EC - Annex V - method B.26 (August 21, 2001), U.S. E.P.A. OPPTS Series 870, Health Effects, Testing Guidelines, N°870.3100 (August 1998) and M.A.F.F. in Japan notification 12 Nousan N°8147 guideline (November 24, 2000). AE C656948, a fungicide of the pyramide family (batch number PFI 0304: a beige powder, 99.0% purity), was administered continuously via dietary administration to separate groups of Wistar rats (10/sex/group) for at least 90 days at concentrations of 50, 200, 1000 and 3200 ppm, corresponding to 3.06 and 3.63, 12.5 and 14.6, 60.5 and 70.1, 204 and 230 mg/kg/day in males and females, respectively. A similarly constituted group of 10 males and 10 females received untreated diet and acted as a control. An additional 10 animals per sex were fed control or high dose test diet for at least 90 days and subsequently observed for

reversibility or persistence of toxic effects after a post-treatment recovery period of at least 28 days. Clinical signs were recorded daily, body weight and food consumption was measured weekly. A detailed physical examination was performed once during the acclimatization phase and weekly throughout the study. All surviving animals (except for animals of the recovery groups) were subjected to a neurotoxicity assessment (motor activity, sensory reactivity and grip strength) during Weeks 11 to 12 of the study. Ophthalmological examinations were performed on all animals during the acclimatization phase and on all surviving animals of the control and high dose groups during Week 13. Urine samples were collected overnight on the week before scheduled necropsy on selected animals. Before scheduled necropsy, a blood sample was collected from the retro-orbital venous plexus on selected animals for hematology and clinical chemistry determinations. In addition, a blood sample was collected into tubes containing heparin from the retro-orbital venous plexus on selected animals for T3, T4 and TSH analysis during Weeks 3 and 13 of the study and on Week 5 of the recovery phase. All animals were necropsied, selected organs weighed and a range of tissues were taken, fixed and examined microscopically. AE C656948 dietary administration to male and female Wistar rats at 3200, 1 000, 200 or 50 ppm for at least 90 days induced no treatment-related mortalities or clinical signs. One male at 1000 ppm and one male at 50 ppm were prematurely sacrificed for humane reasons, with no established treatment relationship.

3200 ppm

Mean body weight was decreased by between 4 and 6% in males and 4 and 8% in females throughout the course of the study, compared to controls. The effect on body weight was primarily due to an initial decrease in mean body weight gain per of 26 and 29% in males and females, respectively, during the first week of treatment, when compared to the control values. Thereafter, mean body weight gain per day was essentially comparable to the controls in both sexes, but mean overall body weight gain was reduced by 9% in males and 17% in females. Mean food consumption was slightly decreased by between 5 and 12% from Days 29 to 90 in females, compared to controls. The neurotoxicity assessment revealed no treatment-related neurotoxic effects in either sex. Ophthalmological examination revealed no abnormalities at the end of the treatment period in either sex. At the hematology evaluation, mean prothrombin time was increased in males, whilst mean platelet and reticulocyte (absolute and percentage) counts were increased in females. In addition, slightly lower mean hemoglobin concentrations were noted in both sexes, in association with

lower mean hematocrit in males and lower mean corpuscular volume and mean corpuscular hemoglobin in females. Clinical chemistry assessment revealed an increase in mean total cholesterol, y-glutamyltransferase, total protein, globulin, inorganic phosphorus and calcium in both sexes, creatinine and urea in males, and triglycerides in females, whereas a decrease was noted in total bilirubin and chloride in both sexes, glucose in males, and alkaline phosphatase and albumin/globulin ratio in females. Urinalysis revealed an increased incidence and severity of cellular casts in males, compared to controls. Hormonal assessment revealed an increase in mean TSH level in both sexes on Week 3, together with an increase in mean T3 and T4 levels in females, whereas at Week 13, only an increase in mean TSH and T3 levels was noted in males. At necropsy, mean absolute and relative liver weights were increased by 53 to 74% and mean absolute and relative thyroid gland weights were slightly increased by 10 to 22% in both sexes. In addition, mean absolute and relative kidney weights were increased by 28 to 36% in males. At the macroscopic examination, enlarged and dark liver and/or prominent lobulation of the liver were observed in both sexes, together with enlarged and/or pale kidneys in males. Microscopic examination revealed treatment-related changes in the liver and thyroid gland in both sexes and in the kidney in males. In the liver, minimal to moderate centrilobular hepatocellular hypertrophy was observed in both sexes, together with minimal to moderate periportal to midzonal hepatocellular macrovacuolation in females. In the thyroid gland, minimal to slight diffuse hypertrophy of follicular cells was seen in the majority of males and in 1/10 females. In the kidney, the incidence and severity of hyaline droplet nephropathy related to the accumulation of $\alpha_{2\mu}$-globulin in the proximal tubules and the incidence of hyaline casts were increased in males. However, hyaline droplet nephropathy is considered not to be toxicologically relevant to man as $\alpha_{2\mu}$-globulin is present only in trace amounts in humans. After 1 month of recovery in the high dose group, mean body weight was still reduced by 7 and 6% in males and females, respectively. Mean food consumption was similar to the control values in both sexes. At the hematology evaluation, only a partial reversibility was observed in mean hemoglobin concentration and associated changes, as mean hemoglobin concentration was still reduced by 4 and 3% in males and females, respectively. Clinical chemistry assessment revealed only a partial reversibility in mean total cholesterol, globulin concentrations and albumin/globulin ratio changes in females. Other hematology and clinical chemistry parameters were comparable to the control values. At the urinalysis, cellular casts were still observed in males, but with a lower incidence and severity than at the end of the dosing phase. Hormonal

assessment showed that all changes detected during the study phase were reversed. At necropsy, mean absolute and relative kidney weights were still increased in males, compared to the controls. No treatment-related changes were detected in liver and thyroid gland weights after 1 month of recovery in either sex. At the macroscopic examination, enlarged kidneys were noted in 2/10 in males. Microscopic examination showed that hyaline droplet nephropathy and hyaline casts persisted in some animals, but that liver and thyroid gland changes noted after 90 days of treatment were reversed after 1 month of recovery.

1000 ppm

A slight decrease of 15% in mean body weight gain per day was noted in females during the first week of treatment, when compared to the control group. Mean food consumption was slightly decreased by between 4 and 9% on most occasions in females, compared to controls. At the hematology evaluation, slightly lower mean hemoglobin concentrations and lower mean hematocrit were noted in males, when compared to the control values. Clinical chemistry assessment revealed an increase in mean total cholesterol in both sexes, an increase in creatinine, inorganic phosphorus and calcium in males, and a decrease in total bilirubin in both sexes and chloride in males. Urinalysis revealed an increased incidence and severity of cellular casts in males, compared to controls. Hormonal assessment revealed an increase in mean TSH level in males on Week 13 only. At necropsy, mean absolute and relative liver weights were increased by 20 to 27% in both sexes. In addition in males, mean absolute and relative kidney weights were increased by 25 to 32%. At the macroscopic examination, enlarged liver and prominent lobulation of the liver were observed in both sexes, together with dark liver and enlarged and/or pale kidneys in males. Microscopic examination revealed treatment-related changes in the liver and thyroid gland in both sexes and in the kidney in males. In the liver, minimal to slight centrilobular hepatocellular hypertrophy was observed in both sexes, together with minimal periportal to midzonal hepatocellular macrovacuolation in females. In the thyroid gland, minimal to slight diffuse hypertrophy of follicular cells was seen in 4/10 males and in 2/10 females. In the kidney, the incidence and severity of hyaline droplet nephropathy, considered not to be toxicologically relevant to man, and the incidence of hyaline casts were increased in males.

200 ppm

Treatment-related changes were noted at the microscopic examination in males only and consisted of minimal centrilobular hepatocellular hypertrophy in the liver of 2/10 animals and minimal diffuse hypertrophy of follicular cells in the thyroid gland of 1/10 animals. In addition in males, a slight increase in incidence and severity of cellular casts was observed in urine, together with the presence of hyaline droplet nephropathy at the microscopic examination of the kidney, but these changes are considered not to be toxicologically relevant to man.

50 ppm

Treatment-related changes consisted only of a slight increase in incidence and severity of cellular casts in urine in males, compared to controls. These changes are considered not to be toxicologically relevant to man.

In conclusion, the dose level of 50 ppm represented the No Observed Adverse Effect Level (N.O.A.E.L.) of AE C656948 in males (equating to 3.06 mg/kg body weight/day), whereas the dose level of 200 ppm represented the No Observed Effect Level (N.O.E.L.) in females (equating to 14.6 mg/kg body weight/day).

[redacted] (2005); AE C656948 – 90-day toxicity study in the mouse by dietary administration; Bayer S.A.S., Bayer CropScience; Report No.: SA 04052; Document No.: M-251136-01.

Summary: This study was conducted according to the O.E.C.D. guideline 408 (1998), E.E.C. Directive

2001/59/EC, Method B.26 (August 21, 2001), U.S. E.P.A. OPPTS Series 870, Health Effects, Testing Guidelines, N°870.3100 (August 1998) and M.A.F.F. in Japan notification 12 Nousan N°8147 guideline (November 24, 2000).

AE C656948, a fungicide of the pyramide family (batch number PFI 0304: a beige powder, 99.0% purity), was administered continuously via dietary administration to separate groups of C57BL/6J mice (10/sex/group) for at least 90 days at concentrations of 30, 150 and 1 000 ppm, corresponding to 5.4 and 6.8, 26.6 and 32, 188 and 216 mg/kg/day in males and females, respectively. A similarly constituted group of 10 males and 10 females received untreated diet and acted as a control group. Clinical signs were recorded daily; body weights and food consumption were measured weekly. A detailed physical examination was performed once during the

acclimatization phase and weekly throughout the study. On the day of necropsy, a blood sample was collected from the retro-orbital venous plexus of each surviving animal for selected clinical chemistry determinations. All animals were necropsied, selected organs weighed and a range of tissues were taken, fixed and examined microscopically.

AE C656948 dietary administration to male and female C57BL/6J mice at 1 000, 150 or 30 ppm for at least 90 days induced no treatment-related mortalities, clinical signs or changes in mean body weight parameters.

At 1000 ppm, mean food consumption was slightly increased by up to 12% on a few occasions in males only, compared to controls. Clinical chemistry assessment revealed a higher mean alanine aminotransferase activity and a slightly lower mean albumin concentration in both sexes, together with a lower mean total cholesterol concentration, a higher mean alkaline phosphatase activity and a tendency towards higher mean aspartate aminotransferase activity in males. At necropsy, mean absolute and relative liver weights were increased by 34 to 38% in males and by 38 to 45% in females. In addition in males, mean absolute and relative adrenal gland weights were increased by 87 to 92%. At the macroscopic examination, enlarged and dark livers were observed in both sexes. Microscopic examination revealed treatment-related changes in the liver and adrenal glands in both sexes. In the liver, minimal to moderate hypertrophy of centrilobular hepatocytes was observed in all animals together with a greater incidence of minimal focal necrosis in 3/10 males and minimal to slight focal necrosis in 6/10 females. In the adrenal glands, a lower incidence of ceroid pigment was noted in males, whilst a greater incidence of minimal to slight cortical vacuolation was observed in females, compared to controls.

At 150 ppm, clinical chemistry assessment revealed a lower mean total cholesterol concentration by 41% in males, compared to the control group. At necropsy, mean absolute and relative liver weights were increased by 9 to 16% in males and by 25 to 28% in females, in correlation with a minimal to slight hypertrophy of centrilobular hepatocytes noted in all males and 5/10 females at the microscopic examination. At 30 ppm, the only treatment-related change consisted of a slight decrease by 30% in mean total cholesterol concentration in males, compared to the control group. In isolation, this change was considered not to be toxicologically relevant.

In conclusion, a dose level of 150 ppm of AE C656948 represented a No Observed Adverse Effect Level (N.O.AE.L.) in males and females (equating to 26.6 and 32.0 mg/kg body weight/day, respectively).

[redacted] (2007); Carcinogenicity study of AE C656948 in the C57BL/6J mouse by dietary administration; Bayer S.A.S., Bayer CropScience; Report No.: SA 05094; Document No.: M-295688-01.

Summary: This study was conducted according to the O.E.C.D. 451 (May, 1981), E.E.C. Directive 88/302/EEC, Method B.32 (November, 1987), U.S. E.P.A. OPPTS Series 870, Health Effects Testing n° 870.4200 (August, 1998) and MAFF in Japan, notification 12 Nousan n° 8147 (November, 2000) guidelines.

The objective of this study was to investigate the oncogenic potential of AE C656948, a fungicide of the pyramide family (batch number Mix-Batch: 08528/0002, a beige powder, 94.5 to 94.7% w/w purity) in the C57BL/6J mouse following continuous dietary treatment for 18 months. In addition, an interim sacrifice was performed after 52 weeks of treatment (to enable assessment of pre-neoplastic changes if required). Groups of 60 male and 60 female C57BL/6J mice were fed diet containing 0, 30, 150 or 750 ppm of AE C656948 for 52 weeks. After 52 weeks, 10 males and 10 females from each group allocated to the chronic phase of the study were necropsied at the scheduled interim sacrifice. The remaining 50 animals/sexy group, allocated to the carcinogenicity phase of the study, continued treatment until the scheduled final sacrifice of the study after at least 78 weeks of treatment. The mean intake of AE C656948 over 18 months was 0, 4.2, 20.9 and 105 mg/kg/day in males and 0, 5.3, 26.8 and 129 mg/kg/day in females, at 0, 30, 150, and 750 ppm respectively. Mortality and clinical signs were checked daily. Additionally, detailed physical examinations including palpation for masses were performed weekly throughout treatment. Body weight and food consumption were measured weekly for the first 13 weeks of the study, then monthly thereafter. Hematology determinations were performed at approximately 12 and 18 months from designated animals. Where possible, blood smears were prepared from moribund animals just before sacrifice. All animals were subjected to necropsy, with selected organs weighed at scheduled interim and final sacrifice. Designated tissues were fixed and examined microscopically. There was no treatment-related effect on mortality, clinical signs or food consumption at any dose level tested.

At 750 ppm

Mean body weight in males was comparable to controls from Week 1 to 26. Between Weeks 30 (study Day 204) to 58 (study Day 400) of the study mean body weight was reduced by up to 5%, and mean cumulative body weight gain by up to 13% over this period from the start of treatment. The effect was statistically significant at most time points during this period ($p<0.05$ or 0.01). Thereafter, mean body weight and mean cumulative body weight gain were comparable to controls until the end of the study. Body weight parameters were not affected in females at 750 ppm. Slightly higher mean platelet counts were noted in males, at Month 13 (+25%, $p<0.01$) and Month 19 (+22%, $p<0.01$), when compared with the controls. At the chronic phase sacrifice (12 months), mean absolute and relative liver weights were increased by between 17 to 25% ($p<0.01$) in males and 28 to 31% ($p<0.01$) in females, whilst mean absolute and relative kidney weights were decreased by between 11 and 17% ($p<0.05$ or 0.01) in both sexes. At the macroscopic examination, enlarged liver was observed in 1/10 males and 2/10 females. A microscopic examination was performed on the thyroid gland of males, as a higher incidence of follicular cell adenomas was observed in males at this dose level, to determine if there were any precursory neoplastic changes in the thyroid gland after 12 months of treatment. Thyroid gland follicular cell hyperplasia was seen in 2/10 males. No treatment-related cause of death was established for the few animals allocated to the carcinogenicity phase (18 months) of the study which died or were humanely sacrificed before the end of the study. At the 18-month terminal sacrifice of the carcinogenicity phase of the study, mean absolute and relative liver weights were increased by between 27 to 31% ($p<0.01$) in males and 35 to 38% ($p<0.01$) in females. In addition, mean kidney to body weight ratio in females was 5% ($p<0.01$) lower than the controls. At the macroscopic examination, enlarged liver was observed in 3/42 males and 30/38 females, and dark liver was noted in 14/42 males and 4/38 females. These findings were correlated with relevant histopathological findings. At the microscopic examination of animals allocated to the carcinogenicity phase (decedents and terminal sacrifice animals), treatment-related changes were seen in the liver, kidney and thyroid gland. The only neoplastic change consisted of a higher incidence of follicular cell adenoma in the thyroid gland in males (7/50), compared with the control group (1/50), this effect was statistically significant ($p<0.05$). Non-neoplastic changes were seen in the liver, kidney and thyroid gland. In the liver, centrilobular to panlobular hypertrophy was observed in both sexes. Centrilobular hepatocellular vacuolation

decreased markedly with concomitant minimal to moderate hepatocellular hypertrophy in males. In addition in males, higher incidences of hepatocellular cholestasis, hepatocellular single cell degeneration/necrosis, interstitial mixed cell infiltrate, eosinophilic inclusion bodies and multinucleated hepatocytes were noted. In females, a higher incidence of eosinophilic foci of altered hepatocytes was observed. In the kidney, a higher incidence and/or severity of bilateral cortical basophilic tubules, hyaline casts(s) and interstitial mononuclear cell infiltrate, together with a higher incidence of glomerular congestion/hemorrhage(s), associated with higher severity of amyloid deposition (mainly observed within glomerular interstitium) was noted in females. In the thyroid gland, a markedly higher incidence of follicular cell hyperplasia was noted in both sexes.

At 150 ppm

At the chronic phase sacrifice (12 months), mean absolute and relative liver weights were increased by between 11 to 15% ($p<0.05$ or 0.01) in males and 17 to 20% ($p<0.05$ or 0.01) in females. At the macroscopic examination, enlarged liver was observed in 2/10 males. At the microscopic examination of the thyroid gland, follicular cell hyperplasia was seen in 2/9 males. At the 18-month terminal sacrifice, mean absolute and relative liver weights were increased by between 14 to 15% ($p<0.01$) in males and 13 to 17% ($p<0.01$) in females. At the macroscopic examination, enlarged liver was observed in 7/40 females. At the microscopic examination of animals allocated to the carcinogenicity phase, treatment-related changes consisted of centrilobular to panlobular hypertrophy observed in the liver of both sexes and a higher incidence of hepatocellular single cell degeneration/necrosis in males, together with a higher incidence of follicular cell hyperplasia observed in the thyroid gland of males.

At 30 ppm

No adverse treatment-related effects were observed at this dose level. The only treatment-related finding was an increase in mean absolute and relative liver weight of between 6 to 8% ($p<0.05$ or 0.01) in males at the 18-month terminal sacrifice. In the absence of additional related microscopic changes, this finding was considered to be non-adverse at this dose level.

In conclusion, dietary administration of AE C656948 over an 18-month period to the C57BL/6J mouse, at a dose level of 750 ppm (equivalent to 105 mg/kg/day in males and 129 mg/kg/day in females) resulted in a higher incidence of follicular cell

adenoma in the thyroid gland in males. Non-neoplastic changes were observed in the liver and thyroid gland in both sexes and in kidney in females at 750 ppm. At 150 ppm (equivalent to 20.9 mg/kg/day in males and 26.8 mg/kg/day in females), non-neoplastic changes were seen in the liver in both sexes and in the thyroid gland in males. The No Observed Adverse Effect Level (NOAEL) was 30 ppm in males (equivalent to 4.2 mg/kg/day), whilst the No Observed Effect Level (NOEL) was 30 ppm in females (equivalent to 5.3 mg/kg/day).

[redacted] (2008); Chronic toxicity and carcinogenicity study of AE C656948 in the Wistar rat by dietary administration; Bayer S.A.S., Bayer CropScience; Report No.: SA 04312; Document No.: M-298339-01.

Summary: This study was conducted according to the O.E.C.D. Guidelines for Testing of Chemicals 453 (May, 1981), E.E.C. Directive 88/302/EEC, Method B.33 (November, 1987), U.S. E.P.A. OPPTS Series 870, Health Effects Testing Guidelines n° 870.4300 (August, 1998) and M.A.F.F. in Japan, notification 12 Nousan n° 8147 (November, 2000) guideline.

The objective of this study was to investigate the chronic toxicity and oncogenic potential of AE C656948 (a fungicide of the pyramide family, batch number Mix-Batch: 08528/0002, a beige powder, >94.5% purity) in Wistar rats following continuous dietary administration over a 24-month period, according to the following design. Groups of seventy males were fed diet containing 30, 150 and 750/375 ppm (due to a high mortality rate in the high dose group, the dose level was decreased from 750 to 375 ppm from study Week 85 onwards), corresponding to 1.20, 6.0 and 29 mg/kg/day over the 24 months, respectively. Groups of seventy females were fed diet containing 30, 150 and 1500 ppm, corresponding to 1.68, 8.6 and 89 mg/kg/day over the 24 months, respectively. After 52 weeks, 10 males and 10 females from each group allocated to the chronic (12 month) phase were necropsied at the scheduled interim sacrifice. The remaining 60 animals/sex/group, allocated to the carcinogenicity (24-month) phase of the study, continued treatment until final sacrifice of the study after at least 104 weeks of treatment. Mortality and clinical signs were checked daily. Detailed physical examinations including palpation for masses were performed at least weekly throughout the study. Body weight was recorded weekly for the first 13 weeks, then approximately every 4 weeks thereafter. Food consumption was recorded twice weekly for the first 6 weeks of the study, then

weekly up to Week 13, then approximately every 4 weeks thereafter. Ophthalmology examinations were performed on all animals during acclimatization and after 12 and 24 months. Hematology, clinical chemistry determinations and urinalysis were performed during months 3, 6, 12, 18 and 24 on selected animals. All surviving animals allocated to the chronic and carcinogenicity phases were subjected to necropsy after a minimum of 52 weeks or 104 weeks of treatment, respectively. Selected organs were weighed and designated tissues sampled and examined microscopically. Decedent animals were subjected to necropsy, designated tissues sampled and examined microscopically.

At 1500 ppm in females

The overall incidence and percentage of mortality was low with no evidence of a treatment-related increase throughout the study. There were no treatment-related clinical signs during the first year of treatment, whereas a higher incidence of hair loss and wasted appearance was noted during the second year, in comparison to the controls. Mean body weight or body weight gain parameters were essentially comparable to the controls throughout the first three months of treatment. Thereafter, mean cumulative body weight gain was lower between Weeks 14 to 26 (-29%, $p<0.01$), 26 to 54 (-15%, not statistically significant) and 54 to 79 (-59%, $p<0.01$) in comparison to the controls, whilst mean body weight was respectively lower by 3, 5, 14 and 12% at Weeks 26, 54, 79 and 102 (statistically significant for most time points), when compared to the controls. Mean food consumption was similar to the controls throughout the study. Ophthalmological examinations revealed abnormal color (pale) of the retinal fundus in 4/67 animals compared to no case in the controls after one year and a higher incidence of small retinal vessels, abnormal color (pale) of the retinal fundus and hyper-reflectivity in retina after two years, in comparison to the controls. There were no toxicologically relevant changes at the hematology evaluation throughout the study. At the clinical chemistry evaluation, the only consistent changes observed throughout the study were slightly higher mean total cholesterol concentrations (+31 to +38% in comparison to the controls). Higher mean triglyceride concentrations were also noted at Months 3 and 6 (+36%), and slightly lower mean glucose concentrations at Months 6, 12 and 18 (-9%, -13%, -16%, respectively). Urinalysis revealed higher incidences of abnormal color of urine (orange to red) throughout the study compared to the controls). At the end of the chronic phase (12 months), mean terminal body weight was slightly lower by 10% (not statistically significant). Mean absolute and relative liver weights were 39 to

54% higher than the controls. This was associated with macroscopic changes (enlarged liver, dark liver and prominent lobulation) and with histological changes including a higher incidence of altered hepatocytes (eosinophilic foci) and hepatocellular brown pigments, focal/multifocal hepatocellular vacuolation, increased number of mitoses, centrilobular to panlobular hypertrophy and hepatocellular single cell necrosis. In the thyroid gland, mean absolute and relative weights were 23 to 38% higher than the controls and follicular cell hypertrophy was noted at the histological examination, in association with a higher incidence and severity of colloid alteration. Dark kidney was found in most animals, compared to no case in the controls, and a higher incidence of tubular golden/brown pigments and of hyaline casts was observed at the histological examination. At the end of the chronic phase, there was no evidence of a treatment-related increased incidence of tumors of any type in any organ. At the end of the carcinogenicity phase (24 months), mean terminal body weight was 11% lower (not statistically significant). Most pathological effects correlated those seen at the end of the chronic phase: mean absolute and relative liver weights were 39 to 56% higher than the controls and were associated with a higher incidence of enlarged liver, dark liver, prominent lobulation, red and white foci on the liver at the macroscopic observation, when compared to the controls. In addition, liver nodules/masses (5/60 females, compared to no case in the controls) were noted and correlated with liver cell carcinoma or adenoma noted at the microscopic examination. Histological changes attributed to the treatment and indicative of marked liver toxicity were also noted, including metabolic, degenerative or proliferative changes. They correspond to the exacerbation of the microscopic findings observed at the end of the chronic phase. In addition in the liver, minimal to slight extramedullary hematopoiesis was observed. In the kidney, a higher incidence of dark kidney, enlarged kidney and irregular surface on the kidney was observed, when compared to the controls. Marked degenerative changes resulting from exacerbation of the microscopic findings noted at the end of the chronic phase were also observed in this organ, together with an increased incidence of tubular golden/brown pigments and collecting ducts hyperplasia. In the thyroid gland, exacerbation of the microscopic findings (follicular cell hyperplasia and/or hypertrophy and colloid alteration) noted at the end of the chronic phase was observed. In the eye, bilateral retinal atrophy was noted, together with a higher incidence of lens degeneration and peripheral bilateral retinal atrophy. Retinal atrophy was characterized by degeneration of the outer plexiform layer, outer nuclear layer and rod/cones lamina. Treatment-related neoplastic changes were observed in

the liver, where a higher incidence of liver cell tumors (carcinoma plus adenoma) was noted, in comparison to the controls. These findings were associated with non-neoplastic/preneoplastic changes and were seen at a dose causing marked hepatocellular toxicity.

At 750/375 ppm in males

During the first year of treatment, 11/70 animals were found dead or were sacrificed prematurely for humane reasons, compared to 6/70 in the controls. The main clinical signs in these early decedent males consisted of soiled fur or anogenital region (3/11), focal swelling (2/11) and wasted appearance (1/11), together with the usual signs associated with morbidity (limited use of hind limbs, reduced motor activity, general pallor). After 2 years of treatment, analysis of the survival rates showed that mortality incidence was increased in the male high dose group, when compared to the controls. No clear cause of death could be established for these early decedent males. There were no treatment-related clinical signs during the first year of treatment, whereas a higher incidence of the usual signs associated with morbidity (prostration, general pallor and soiled anogenital region) was noted during the second year, in comparison to the controls. Mean body weight or body weight gain parameters and mean food consumption were essentially comparable to the controls throughout the study, except for a slight reduction in mean food consumption by up to 7% ($p<0.01$ or $p<0.05$) between study Days 18 to 39. At the ophthalmological examinations, there were no treatment-related changes after one year, whereas a higher incidence of cornea! opacity, oedema of the cornea, nuclear opacity of lens, small retinal vessels and abnormal color (pale) of the retinal fundus was noted after two years, in comparison to the controls. There were no treatment-related changes at the hematology and clinical chemistry evaluations throughout the study. Urinalysis revealed a higher incidence and severity of cellular casts at Months 3 and 6, when compared to the controls. This change was found to be reversible, as no cellular casts were noted at Months 18 and 24. At the end of the chronic phase (12 months), mean terminal body weight was unaffected by the treatment, whilst mean absolute and relative liver weights were higher by between 17 to 18% and mean absolute and relative kidney weights by 28%, in comparison to the controls. At the macroscopic observation, enlarged liver was found in 1/10 animals and prominent lobulation on the liver in 3/10 animals, compared to no case in the controls. In addition, pale kidney, enlarged kidney or irregular surface on the kidney were found in some animals, compared to no case in the controls. Histological examination revealed lexicologically relevant

changes in the liver, kidney and thyroid gland, in comparison to controls. In the liver, the incidence of altered hepatocytes (eosinophilic foci) was higher. In addition, centrilobular to panlobular hypertrophy was observed. In the kidney, chronic progressive nephropathy was noted. This change is a combination of thickened basement membranes (tubular and glomerular), basophilic tubules and hyaline casts with a variable inflammatory cell infiltrate. In addition, a higher incidence of hyaline droplets and of bilateral basophilic tubules was noted. In the thyroid gland, follicular cell hypertrophy was noted, in association with a higher incidence and severity of colloid alteration. At the end of the chronic phase, there was no evidence of a treatment-related increased incidence of tumors of any type in any organ. At the end of the carcinogenicity phase (24 months), mean terminal body weight was 7% lower (not statistically significant) and mean absolute and relative liver weights were 5 to 12% higher, in comparison to the control group. At the macroscopic observation, a higher incidence of enlarged liver and white foci on the liver were found, together with a higher incidence of enlarged kidney and irregular surface on the kidney, when compared to the controls. Histological examination revealed toxicologically relevant changes in the liver, kidney, thyroid gland, and secondary effects in the testis and stomach, in comparison to the controls. In the liver and thyroid gland, changes resulting from exacerbation of the findings noted at the end of the chronic phase were observed. In the kidney, marked degenerative changes resulting from exacerbation of the microscopic findings noted at the end of the chronic phase were observed, together with a higher incidence of tubular hypertrophy, collecting ducts hyperplasia and hyaline droplets. In the testis, a higher incidence of arteritis and periarteritis was noted. This vascular change was isolated (not found in sensitive tissues like aorta, mesenteric arteries) and is likely explained by secondary hypertensive changes due to increased severity and incidence of chronic nephropathy. Therefore this change in the testis was considered not to be a direct effect of the treatment. In the stomach, a higher incidence of regenerative non glandular hyperplasia was noted. This minor change was mainly observed in animals found prematurely dead and was attributed to a secondary stress due to morbidity. Therefore, its increased incidence was clearly linked to the increased mortality rate in this male high dose group. No treatment-related neoplastic changes were observed.

At 150 ppm

There were no treatment-related clinical signs throughout the study in either sex. Mean body weight or body weight gain parameters and mean food consumption were

unaffected by treatment in either sex over the two years of the study. At the ophthalmological examinations, there were no treatment related changes after one year, whereas a higher incidence of corneal opacity, oedema of the cornea, nuclear opacity of lens and small retinal vessels was noted in males after two years, in comparison to the controls. There were no toxicologically relevant changes at the hematology and clinical chemistry evaluations throughout the study. Urinalysis revealed a higher incidence and severity of cellular casts at Months 3 and 6 in males, when compared to the controls. This change was found to be reversible, as no cellular casts were noted in this male group at Months 12, 18 and 24. At the end of the chronic phase (12 months), mean terminal body weights and mean organ weights were unaffected by treatment in either sex. No treatment-related changes were noted at the macroscopic observation. Histological examination revealed toxicologically relevant changes in the liver, kidney and thyroid gland in males only, in comparison to controls. In the liver, centrilobular to panlobular hypertrophy was observed. In the kidney, chronic progressive nephropathy was noted, together with a higher incidence of hyaline droplets. In the thyroid gland, follicular cell hypertrophy was observed. At the end of the carcinogenicity phase (24 months), mean terminal body weights and mean organ weights were unaffected by treatment in either sex, with the exception of a slight increase by between 8 to 12% in mean absolute and relative liver weights in males, when compared to the controls. At the macroscopic observation, a higher incidence of enlarged kidney was noted in males from the unscheduled sacrifice group only, when compared to the controls. Histological examination revealed toxicologically relevant changes in the liver, kidney, thyroid gland and testis, in comparison to the controls. In the liver, changes resulting from exacerbation of the findings noted at the end of the chronic phase were observed in males. In the kidney, a higher incidence and severity of chronic progressive nephropathy and a higher incidence of tubular hypertrophy and tubular dilatation were observed in males. In the thyroid gland, follicular cell hypertrophy was observed in males, whereas a higher incidence of colloid alteration was noted in females. In the testis, a higher incidence of arteritis and periarteritis was noted. This change was considered not to be a direct effect of the treatment. No treatment-related neoplastic changes were observed at this dose level in either sex.

At 30 ppm

No toxicologically relevant changes were noted throughout the course of the study in either sex for any of the parameters evaluated.

In conclusion, there was no evidence of a treatment-related increased incidence of tumors of any type in any organ, with the exception of a higher incidence of liver cell tumors (carcinoma plus adenoma) in the female high dose group only at the end of the carcinogenicity phase, in comparison to the controls. Over a 24-month period of dietary administration with AE C656948 to the Wistar rat, the dose level of 30 ppm represented a No Observed Adverse Effect Level (N.O.A.E.L.) in males (equivalent to 1.20 mg/kg body weight/day) and a No Observed Effect Level (N.O.E.L.) in females (equivalent to 1.68 mg/kg body weight/day).

[redacted]. (2008); Fluopyram (AE C656948) - 7-day mechanistic study in the female Wistar rat by dietary administration; Bayer S.A.S., Bayer CropScience; Report No.: SA 07323; Document No.: M-299274-01.

Summary: The objective of the study was to investigate the effects of fluopyram (AE C656948) on the rat liver in order to clarify its mode of action especially in view of the liver tumors observed in the female rats at the end of the carcinogenicity study. Fluopyram (AE C656948), a fungicide currently being developed by BayerCropScience (Batch number: Mix Batch: 08528/0002: 94.7% w/w purity), was administered continuously in the diet to groups of female Wistar rats (15/group) for 7 days at the concentration of 3000 ppm (equivalent to 193 mg/kg body weight/day). A similarly constituted group received untreated diet and acted as a control group. Animals were observed daily for mortality and clinical signs. Physical examinations were performed at least weekly. Body weight and food consumption were recorded at the end of the 7-day treatment period. Hepatic cell proliferation was assessed by administration of 5-bromo-2'-deoxyuridine (BrdU, an analogue of thymidine) to all animals in the drinking water for 7 days before sacrifice. Water consumption was measured during BrdU administration period. All animals were subjected to necropsy. Brain and liver were weighed. Selected portions of the liver were fixed for conventional histopathological examination and cell proliferation measurement. The remaining portions of the liver of 10 females from each group were homogenized for microsomal preparations in order to determine total cytochrome P-450 content and cytochrome P-450 isoenzyme and UDPGT activities. Fluopyram (AE C656948) at 3000 ppm in the diet had no effect on body weight parameters. There was no evidence of a treatment-related effect on clinical signs, food or water consumption. Mean absolute and relative liver weights were increased by between 40 to 43% when compared to the controls (statistically significant: $p<0.01$). This increase was

associated with macroscopic findings (enlarged and dark livers) in 13/15 females compared to no instance in the controls. At histological examination, minimal to slight centrilobular to panlobular hepatocellular hypertrophy was found in all treated animals. A markedly decreased incidence of periportal vacuolation was also noted. Assessment of cell proliferation in the liver revealed a 4 times higher mean BrdU labeling index in both the perilobular and centrolobular areas of the hepatic lobule in treated animals, when compared to the controls. Assessment of total cytochrome P-450 content and microsomal proteins revealed a slight increase in total P-450 content and in mean EROD activity, a moderate increase in mean PROD and BROD activities and an important increase in mean UDPGT activity (all statistically different from the control group ($p<0.01$)). These data showed that AE C656948 has the ability to induce moderately phenobarbital-inducible hepatic enzymes (BROD, PROD and UDPGT) as well as a liver hypertrophy and cell proliferation in the liver.

[redacted] (2008); Phenobarbital - 7-day mechanistic study in the female Wistar rat by gavage; Bayer S.A.S., Bayer CropScience; Report No.: SA 07325; Document No.: M-299491-01.

Summary: The objective of this study was to evaluate the liver changes caused by phenobarbital to female Wistar rat following administration by gavage for 7 days. Phenobarbital (sodium salt, batch: 06100228, white crystalline powder, purity 99.6%) was administered by gavage to groups of female Wistar rats (15/group) for 7 days at the concentration of 80 mg/kg body weight/day. A similarly constituted group received untreated diet and acted as a control group. Animals were observed daily for mortality and clinical signs. Physical examinations were performed at least weekly. Body weight and food consumption were recorded at the end of the 7-day treatment period. Hepatic cell proliferation was assessed following administration of 5-bromo-2'-deoxyuridine (BrdU, an analogue of thymidine) to all animals in the drinking water for 7 days before sacrifice by determining the BrdU labeling index. Water consumption was measured during BrdU administration period. All animals, except animal found dead during the study, were subjected to necropsy. Brain and liver were weighed. Selected portions of the liver were fixed for conventional histopathological examination and cell proliferation measurement. The remaining portions of the liver of 10 females from each group were homogenized for microsomal preparations in order to determine total cytochrome P-450 content and cytochrome P-450 isoenzymes and UDPGT activities. Administration of phenobarbital at 80 mg/kg/day for 7 days

caused a reduced motor activity in all animals tested. One animal was found dead on Study Day 5. There was a slight effect on body weight with an overall mean absolute body weight gain of 0 g compared to 7 g in the control group. At necropsy, there was no relevant change in mean terminal body weights when compared to controls. Mean absolute and relative liver weights were increased by between 19 and 22% when compared to the controls (statistically significant: $p<0.01$). At macroscopic examination, dark liver was found in 5/14 females and enlarged liver in 3/14 females compared to no case in the controls. At histological examination, minimal to slight centrilobular to panlobular hepatocellular hypertrophy was found in all treated animals. A decreased incidence of periportal vacuolation was also noted. Assessment of cell proliferation in the liver revealed a 2 to 3 times higher mean BrdU labeling index in the perilobular area and in the centrilobular area in the treated animals compared to the controls. Assessment of total cytochrome P-450 content and hepatic enzymes activities revealed a moderate increase in total P-450 content and in mean PROD, BROD and UDPGT activities (all statistically different from the control group $p<0.01$). These data indicate that phenobarbital has the ability to induce hepatic enzymes like total cytochrome P-450, PROD, BROD and UDPGT activities as well as a liver hypertrophy and cell proliferation in the liver.

[redacted] (2008); AE C656948 (Fluopyram) - In vitro studies on the potential interactions with thyroid peroxidase-catalyzed reactions; Bayer HealthCare AG; Report No.: AT04481; Document No.: M-299276-01.

Summary: To investigate a potential effect of AE C656948 on thyroid hormone synthesis at the level of thyroid peroxidase (TPO), interactions of this compound with TPO-catalyzed reactions were studied in vitro using solubilized hog thyroid microsomes as an enzyme source. Amitrole and Ethylenethiourea (ETU) served as positive control substances Amitrole, a potent inhibitor of thyroid peroxidase, strongly inhibited TPO-catalyzed oxidation of guaiacol and formation of iodine. About 50% inhibition was observed in the presence of 1 uM Amitrole for guaiacol oxidation and in the presence of 0.1 uM Amitrole for iodine formation. Ethylenethiourea, which is not a TPO inhibitor, but a trap of the iodinating intermediate generated by TPO from iodide, temporarily suppressed iodine formation. In contrast, AE C656948 did not affect TPO-catalyzed guaiacol oxidation up to 300 uM, the highest concentration tested. Similarly, TPO-catalyzed iodine formation was

not affected by 300 uM AE C656948. These findings strongly suggest that AE C656948 does not affect thyroid hormone synthesis at the level of TPO.

[redacted] (2008); AE C656948 - Mechanistic 14-day toxicity study in the mouse by dietary administration (hepatotoxicity and thyroid hormone investigations); Bayer S.A.S., Bayer CropScience; Report No.: SA 07215; Document No.: M-299522-01.

Summary: AE C656948, a fungicide of the pyramide family (batch number: Mix-batch: 08528/0002: light beige powder, 94.7% purity), was administered continuously via the diet to 2 groups of 15 male C57BL/6J mice for 3 or 14 days at a concentration of 2000 ppm, equating approximately to 308 and 314 mg/kg body weight/day, respectively. Two similarly constituted groups of 15 males received untreated diet and acted as a control groups. Clinical signs were recorded daily, body weight and food consumption was measured weekly. A detailed physical examination was performed weekly. On study Day 4 and Study Day 15, before necropsy, blood samples were taken for hormone levels measurement (T3, T4 and TSH). The liver and the brain were weighed and the liver and the thyroid gland were sampled for microscopic examination. In addition, hepatic cytochrome P-450 isoenzymes and UDPGT activities were also measured using 4-nitrophenol as substrate. There were no mortalities and no treatment-related findings in term of clinical signs and body weight parameters during the course of the study.

After 3 days of exposure, at 2000 ppm, mean T3 level was not changed as compared to the untreated control group whereas mean T4 level was decreased (-30%) and mean TSH level was increased (+18%) when compared to controls. Mean absolute and relative liver weights were increased by approximately 60% when compared to control animals. At macroscopic observation, enlarged liver was found in all the treated animals. At microscopic examination, diffuse centrilobular to panlobular hepatocellular hypertrophy and an increased number of mitoses were seen in all the examined treated animal livers. Moreover, hepatocellular single cell necrosis was observed in 1 out the 5 treated animal livers examined. No significant microscopic change was observed in the thyroid gland. Total cytochrome P-450 content was highly increased (+116%) by the treatment. EROD activities were marginally increased (+235%) whereas PROD and BROD activities were markedly increased

(respectively +2809% and +8717%) when compared to controls. No significant changes were observed in UDPGT.

After 14 days of exposure at 2000 ppm, mean T3 level was not changed whereas mean T4 level was decreased (-27%) and mean TSH level was increased (+7%) when compared to controls. Mean absolute and relative liver weights were increased by approximately 60% when compared to the one of control animals. At macroscopic observation, enlarged liver was found in 13 out of 15 the treated animals compared to no instances in the controls moreover dark liver was observed in 14 out the 15 treated animals compared to only in 1 control animal. At microscopic examination, diffuse centrilobular to panlobular hepatocellular hypertrophy was seen in all the examined treated animal livers. Moreover, hepatocellular single cell necrosis was observed in 4 out the 5 treated animal livers examined. No significant microscopic change was observed in the thyroid gland. Total cytochrome P-450 content was moderately increased (+71%) by the treatment. EROD activities were marginally increased (+165%) whereas PROD and BROD activities were markedly increased (respectively +2163% and +9061%) when compared to controls. No relevant changes were observed in UDPGT activities.

In conclusion, this study demonstrates that AE C656948 dietary administration at a nominal concentration of 2000 ppm in the C57BL/6J mouse for 3 and 14 days induced Phenobarbital-like P-450 hepatic enzymatic activities and a modification of the pituitary and thyroid hormone levels.

[redacted] (2008); Phenobarbital - Mechanistic 14-day toxicity study in the mouse by oral gavage (hepatotoxicity and thyroid hormone investigations); Bayer S.A.S., Bayer CropScience; Report No.: SA 07326; Document No.: M-299521-01.

Summary: Phenobarbital, a reference hepatoxic compound (batch number: 06100228: white powder, 99.6% of purity), was administered daily by gavage to 2 groups of 15 female C57BL/6J mice for 3 or 14 days at a dose level of 80 mg/kg/day. Two similarly constituted groups of 15 females received 0.5% methycellulose for 3 or 14 days and acted as a control groups. Clinical signs were recorded daily, body weight and food consumption was measured weekly. A detailed physical examination was performed weekly. Before necropsy, blood samples were taken for thyroid and pituitary gland hormone analyses (T3, T4 and TSH). At final sacrifice times, liver and

brain were weighed and liver and thyroid gland sampled for the assessment of morphological changes. In addition, hepatic cytochrome P-450 and UDPGT isoenzyme activities were assessed. There were no mortalities and no treatment-related findings in term of clinical signs during the course of the study.

After 3 days of exposure, at terminal sacrifice, mean T3 and T4 levels were decreased (respectively -10% and -27%) and mean TSH level was not affected when compared to controls. At necropsy mean absolute and relative liver weights was increased 4% to 11% when compared to control animals. At macroscopic observation, enlarged liver was found in 1/15 of the treated animals moreover dark liver was observed in 6/15 treated animals compared to no instances in the controls. At microscopic examination, diffuse centrilobular to panlobular hepatocellular hypertrophy were seen in 4/5 examined treated animal livers and an increased number of mitoses in 3/5 treated animal livers. Total cytochrome P-450 content was highly increased (+146%) by the treatment. EROD activity was marginally increased (+297%) whereas PROD and BROD activities were markedly increased (respectively +1381 and +4930%) when compared to controls. No significant change was observed in UDPGT activity when measured with 4-nitrophenol as substrate.

After 14 days of exposure at terminal sacrifice, mean T3 level was not changed whereas mean T4 level was decreased (-19%) and mean TSH level was increased (+9%) when compared to controls. At necropsy mean absolute and relative liver weights was increased 21% to 23% when compared to control animals. At macroscopic observation, enlarged liver was found in 12/15 the treated animals compared to only in 1 control animal moreover dark liver was observed in 4/15 treated animals compared to no instances in the controls. At microscopic examination, diffuse centrilobular to panlobular hepatocellular hypertrophy were seen in all the examined treated animal livers.

Total cytochrome P-450 content was slightly increased (+36%) by the treatment. EROD activity was slightly increased (+375%) whereas PROD and BROD activities were markedly increased (respectively +1345% and 2844%) when compared to controls. No significant change was observed in UDPGT activities when measured with 4-nitrophenol as substrate.

In conclusion, this study demonstrate that phenobarbital administration at a dose level of 80 mg/kg body weight/day in the C57BL/6J mouse for 3 and 14 days induced a

hepatic total cytochrome P-450, PROD and BROD activities induction and a modification of the pituitary and thyroid hormone levels.

[redacted] (2008); AE C656948 - Mechanistic 3-day toxicity study in the male mouse (pharmacokinetic investigations of the clearance of intravenously administered ^{125}I-thyroxine); Bayer S.A.S., Bayer CropScience; Report No.: SA 08159; Document No.: M-308369-01.

Summary: AE C656948, a fungicide (batch number: Mix-batch: 08528/0002: light beige powder, 94.7% purity) was administered continuously via the diet at a dose level of 2000 ppm to a group of 5 male C57BL/6J mice for 3 days, a similarly constituted group of 5 males received untreated diet and acted as a control group. In addition, a further group of 5 males received 80 mg/kg/day Phenobarbital (batch number: 06100228: white powder, 99.6% purity) by oral gavage for a 3 day period. Phenobarbital acted as a reference compound known to induce an increase in T4 clearance in the mouse through induction of T4 glucoronidation (6, 7). On Study Day 4 each animal received by intravenous injection via the tail 250 ul of diluted ^{125}I-Thyroxine solution. Approximately 3 hours post-administration with ^{125}I-Thyroxine, each animal received 0.1 mg of NaI in 250 ul of 0.9% sterile saline by intraperitoneal injection. A blood sample was collected from the retro-orbital venous plexus of each animal after 1 hour 20 minutes, 2, 4, 6 and 24 hours post ^{125}I-Thyroxine administration. The level of ^{125}I radioactivity in each sample was measured using a Cobra gamma scintillation counter. The rate of ^{125}I radioactivity was indicative of the rate of Thyroxine (T4) clearance from the blood. Animals were checked daily for mortality and clinical signs. Body weights were recorded on Study Days 1 and 4. Due to technical difficulties encountered with the intravenous injection of ^{125}I-Thyroxine, Thyroxine (T4) clearance data for 9 animals only (5 control animals, 1 AE C656948 treated animal and 3 Phenobarbital treated animals) was obtained from this first group of animals (subgroup 1). Consequently, 5 additional animals were incorporated onto the study (subgroup 2). One animal acted as a control and received untreated diet, whilst the remaining 4 animals were treated with 2000 ppm AE C656948. The results obtained in the main and the complementary experiments were combined. There were no mortalities or clinical signs during the course of the study. There was no statistically significant effect on body weight.

The results show that following an intravenous of ^{125}I-Thyroxine the radioactivity level in the blood of the AE C656948 treated animals was lower than that in the blood of the corresponding control animals. This decrease in the level of radioactivity in the blood of AE C656948 treated animals was observed at all time-points examined and reflects a more rapid clearance of Thyroxine in these animals over a 24 hour period, compared with the controls. A similar response to that observed with AE C656948 treated animals was seen in animals treated with the reference compound Phenobarbital.

In conclusion, these results indicate that the clearance of Thyroxine from the blood of animals treated with 2000 ppm AE C656948 was increased when compared to control animals over a 24 hour period. In addition, the response observed with AE C656948 treated animals was similar to that noted in animals treated with 80 mg/kg/day Phenobarbital.

[redacted] (2008); AE C656948 - Mechanistic 3-day toxicity study in the male mouse (QPCR investigations of gene transcripts in the liver); Bayer S.A.S., Bayer CropScience; Report No.: SA 08151; Document No.: M-308073-01.

Summary: AE C656948, a fungicide (batch number: Mix-batch: 08528/0002: Light beige powder, 94.7% purity) and Phenobarbital (batch number: 06100228: White powder, 99.6% purity), were administered daily to 2 groups of 10 male C57BL/6J mice for 3 days at dose levels of 2000 ppm and 80 mg/kg/day, respectively. One similarly constituted group of 10 males received control diet for 3 days and acted as a control group. Clinical signs were recorded daily and body weight was measured on Study Days 1 and 4. At final sacrifice, liver was weighed and sampled for gene expression analyses by quantitative Polymerase Chain Reaction (qPCR). There were no mortalities or body weight effects during the course of the study. Animals treated with Phenobarbital showed reduced motor activity throughout the treatment period.

At necropsy, mean absolute and relative liver weights were increased by 60% to 61% for AE C656948 treated animals and by 17% to 19% for Phenobarbital treated animals, when compared to the control animals. Quantitative PCR analyses of transcripts of genes known to be implicated in the hepatic inactivation of thyroid hormones revealed in AE C656948 treated animals an up-regulation of sulfotransferase transcripts (from +92% to +463%, $p<0.01$) and UDP

glucoronosyltransferase transcripts (from+173% to +273%, p<0.01). Similarly, an up-regulation of sulfotransferase transcripts *Sultlal* and *Sultn* (+62% and +96%, respectively, p<0.01) and UDP glucoronosyltransferase transcripts (from +82% to +119%, p<0.01) was observed in Phenobarbital treated animals.

In conclusion, this study demonstrates that AE C656948 at 2000 ppm and Phenobarbital at 80 mg/kg body weight/day administered to the C57BL/6J mouse for 3 days both induced an up-regulation of the sulfotransferase and UDP glucoronosyltransferase gene transcripts in the liver. These transcripts are known to encode enzymes that inactivate T3 and T4 via glucoronide and sulfate derivatives.

[redacted] (2009); AE C656948 - Definitive mechanistic 4-day toxicity study in the male mouse (pharmacokinetic investigations of the clearance of intravenously administered ^{125}I-thyroxine); Bayer S.A.S., Bayer CropScience; Report No.: SA 08288; Document No.: M-328662-01.

Summary: The objective of this mechanistic study was to determine the potential effects of AE C656948 in the male mouse on the clearance of Thyroxine (T4) in the blood following continuous dietary administration for 4 days. AE C656948, a fungicide (batch number: Mix-batch: 08528/0002: light beige powder, 94.7% purity) was administered continuously via the diet at a dose level of 2000 ppm to a group of 8 male C57BL/6J mice for 4 days, a similarly constituted group of 8 males received untreated diet and acted as a control group. In addition, a further group of 8 males received 80 mg/kg/day Phenobarbital (batch number: 06100228: white powder, 99.6% purity) by oral gavage for a 4 day period. Phenobarbital acted as a reference compound known to induce an increase in T4 clearance in the mouse through induction of T4 glucuronidation and sulfonation. On Study Day 5 each animal received 250 ul of diluted I25l-Thyroxine solution in 0.9% sterile saline by intravenous injection via the tail vein. Approximately 3 hours after administration of ^{125}I-Thyroxine, each animal received 0.1 mg of NaI in 250 ul of 0.9% sterile saline by intraperitoneal injection. A blood sample was collected from the retro-orbital venous plexus of each animal 40 minutes, 1.5, 4, and 24 hours post ^{125}I-Thyroxine administration. The level of ^{125}I radioactivity in each sample was measured using a gamma scintillation counter. The level of ^{125}I radioactivity was indicative of the rate of Thyroxine (T4) clearance from the blood. Animals were checked daily for mortality and clinical signs. Body weights were recorded on Study Days 1 and 5.

There were no clinical signs during the course of the study. One animal was sacrificed after intravenous injection of the radiolabeled Thyroxine due to a technical problem during the injection. There was no statistically significant effect on body weight.

The results show that following an intravenous injection of ^{125}I-Thyroxine, the radioactivity level in the blood of the AE C656948 treated animals was lower than the blood radioactivity level of the concurrent control animals. This decrease in the level of radioactivity in the blood of AE C656948 treated animals was observed at all time-points examined and is considered to reflect a more rapid clearance of Thyroxine in these animals over a 24 hour period, compared with the controls. A similar response to that observed with AE C656948 treated animals was seen in animals treated with the reference compound Phenobarbital.

In conclusion, these results indicate that the clearance of Thyroxine from the blood of animals treated with 2000 ppm AE C656948 was increased when compared to control animals over a 24 hour period. In addition, the response observed with AE C656948 treated animals was similar to that noted in animals treated with 80 mg/kg/day Phenobarbital.

[redacted] (2011); Fluopyram – Mechanistic 3-day toxicity study in the mouse by oral gavage (thyroid hormone investigations); Bayer S.A.S., Bayer CropScience; Report No.: SA 10241; Document No.: M-408352-01.

Summary: Fluopyram, a fungicide of the pyramide family (batch number: Mix-batch: 08528/0002: Light beige solid, 94.7% purity), was administered continuously by oral gavage to 2 groups of 15 male C57BL/6J mice for 3 days at 100 and 300 mg/kg body weight/day. Phenobarbital (batch number: 06100228: White powder, 99.6% purity) was administered continuously by oral gavage to one group of 15 male C57BL/6J mice for 3 days at 80 mg/kg body weight/day. A similarly constituted group of 15 males received the vehicle alone (0.5% methylcellulose) and acted as a control group. Clinical signs were recorded daily and body weight was measured on Day 1 and Day 3. A detailed physical examination was performed once during the treatment period. On study Day 4, before necropsy, blood samples were taken for hormone levels measurement (T4 and TSH). The gall bladder was taken and the bile was collected for T4 level measurement. The pituitary gland was sampled for qPCR analysis of the *Tsh*

transcript. There were no mortalities and no treatment-related findings in term of clinical signs and body weight parameters during the course of the study.

In the Fluopyram treated animals, after 3 days of exposure, at 100 and 300 mg/kg/day, mean T4 levels in the plasma were decreased (-26% and -34%, $p<0.01$) whilst mean TSH levels in the plasma were not changed when compared to controls. An increase in the levels of *Tsh* transcript (+21% n.s.; +49%, $p<0.01$) were observed in the pituitary gland.

In the Phenobarbital treated animals, after 3 days exposure at 80 mg/kg/day, mean T4 levels in the plasma were decreased (-38%, $p<0.01$), whilst mean TSH levels in the plasma were not changed when compared to the controls. An increase in the levels of *Tsh* transcript (+46%, $p<0.01$) was observed in the pituitary gland.

In conclusion, this study demonstrates that Fluopyram administration by oral gavage at nominal concentrations of 100 and 300 mg/kg/day, in the C57BL/6J mouse for 3 days, induced a decrease in plasma T4 levels associated with an increase in the levels of *Tsh* transcript (beta subunit) in the pituitary gland, whereas no increase of plasma TSH levels were detected.

[redacted] (2011); Fluopyram - Mechanistic investigations in the female rats by dietary administration for up to 7 days; Bayer S.A.S., Bayer CropScience; Report No.: SA 10240; Document No.: M-408029-01.

Summary: Fluopyram, (batch number: Mix-batch: 08528/0002: a light beige solid, 94.7% w/w), a fungicide, was administered daily in the diet to groups of adult female Wistar rats (30 rats/dose level) for 3 days (Subgroup 1; n = 15/group) or for 7 days (Subgroup 2; n = 15/group) at dose levels of 30, 75, 150, 600 and 1500 ppm. These doses equated to 2.4, 6.2, 12.0, 46.1 and 117.6 mg/kg/day, respectively for those animals sacrificed after 3 days of treatment. For those animals sacrificed following 7 days of treatment the doses corresponded to 2.3, 5.6, 11.6, 44.1 and 118.5 mg/kg/day, respectively. A group of animals dosed by oral gavage with 80 mg/kg/day Phenobarbital (a CAR/PXR nuclear receptor activator) acted as a positive control for the parameters investigated in this present study. A control group received untreated diet. Animals were observed daily for mortality and clinical signs. Physical examinations were performed weekly. Body weight and food consumption were recorded once weekly. All animals were necropsied in the morning following three or seven days of treatment, the liver was weighed and sampled for investigation of

several parameters. Specifically, samples were fixed and examined microscopically. Additional slides were stained for Ki67 for cell proliferation determinations. The duodenum was also sampled and used as a positive control tissue for the cell proliferation investigations. Small portions of the liver were frozen in liquid nitrogen and used for gene expression investigations. The remaining portions of the liver from 5 randomly chosen females/group that were sacrificed after 7 days treatment were homogenized for microsomal preparations in order to determine cytochrome P-450 and UDPGT isoenzyme profiles.

There were no clinical signs and no effects on food consumption or body weight parameters for any of the groups treated with Fluopyram (both sacrifice times). Clinical signs consisting of reduced motor activity were, however, recorded for all females dosed with Phenobarbital and some of these females had ocular discharge in one or both eyes. In addition, mean absolute body weight gain was reduced by 23% (not statistically significant) for those females dosed with Phenobarbital for 7 days. Fluopyram induced treatment-related changes in all liver parameters investigated. The number of parameters affected and also the magnitude of the responses were dose related as described below.

At 30 ppm, there were no treatment-related changes in any of the parameters measured at either of the sacrifice times.

At 75 ppm, a marginal but statistically significant increase in the expression of *Cyp3a3* was recorded both after three (+48.4%; $p \leq 0.05$) and seven days (+95%; $p \leq 0.01$) of treatment. This increase in gene expression was, however not associated with any increase in enzyme (BROD) activity following seven days of treatment.

At 150 ppm, a slight, though significant, increase in hepatic cell proliferation (centrilobular and perilobular) was observed. This effect was similar in magnitude at both sacrifice times. In addition, increased gene expression of *Cyp2b1* as well as *Cyp3a3* was recorded at this dose level and for both sacrifice times. Following 7 day treatment the gene expression of *Cyp1a1* was statistically significantly increased. These increases in gene expression for the Phase I enzymes were not associated with increased PROD, BROD or EROD enzyme activity following seven days of treatment.

At 600 ppm, the increased cell proliferation was more marked than that already observed at 150 ppm. For example, the % change (compared to controls) in global cell proliferation at 3 days was 266.6% compared to 86.0% at 150 ppm and at 7 days

it was 191.7% compared to 106% at 150 ppm. A slight but statistically significant increase in liver weight (absolute and relative to body weight) was recorded following 3 days treatment and in one female minimal centrilobular to panlobular hepatocellular hypertrophy was observed following 7 days treatment. Statistically significant increases in enzyme activity (BROD, PROD and both isoforms of UDPGT) were also recorded following 7 days treatment. Statistically significant increases were recorded at both sacrifice times for the gene expression of Phase I (*Cyp2b1*; *Cyp3a3*) and Phase II enzymes (*Udpgtr2*; *Gstm4* and *Ephx1*). *Gsta2* was significantly increased following 7 days treatment. *Cyp1a1* gene expression was statistically significantly increased at both sacrifice times but was not associated with any enzyme activity at this dose level.

At 1500 ppm, the effects on cell proliferation were even more marked than those recorded at 600 ppm. Specifically, the % change (compared to controls) in global cell proliferation at 3 days was 551.2% compared to 266.6% at 600 ppm and at 7 days it was 256.6% compared to 191.7% at 600 ppm. In addition, an increased number of mitoses was observed in 4/15 females dosed for 3 days treatment with Fluopyram. Furthermore a slight, though statistically significant increase in the expression of *Tacstd1* (a marker for cell proliferation; 3 day treatment only) and *Gadd45b* (a marker for apoptosis, following 3 and 7 days treatment) was recorded. Significant increases in mean absolute and relative liver weight were recorded at both time points, which could be associated with the centrilobular to panlobular hypertrophy observed in 6/15 females following 3 day treatment (minimal) and in 14/15 females following 7 days treatment (minimal to slight). The increases in enzyme activity observed at 600 ppm were more marked at 1500 ppm with statistically significant increases in EROD as well as total P450 content also being recorded at this dose level.

Phenobarbital was used as a positive control for the various parameters measured in the present study and as such induced changes in the liver pertinent for a compound that activates the CAR/PXR nuclear receptors. Thus, increased cell proliferation was observed following both 3 and 7 days treatment, with the effects being more apparent in the centrilobular region (+217.7% and +603.4% increases compared to controls at 3 and 7 days, respectively) than in the perilobular region (+58.0% and +54.7% increases compared to the controls at 3 and 7 days, respectively). In addition an increased number of mitoses was observed in 3/15 females at both time points. Furthermore Gadd45b (a marker for apoptosis, following 3 and 7 days treatment)

gene expression was statistically significantly increased at both time points. Liver weight (absolute and relative to brain and body weight following 3 days treatment; relative to body weight only following 7 day treatment) was statistically significantly increased following Phenobarbital treatment, which could be associated with the centrilobular to panlobular hypertrophy observed in 3/15 females following 3 day treatment (minimal) and in 9/15 females following 7 days treatment (minimal to slight). BROD, PROD and UDPGT-nitrophenol activity were statistically significantly increased due to 7 days treatment with Phenobarbital. Furthermore, statistically significant increases in the gene expression of *Cyp2b1*, *Cyp3a3* and *Udpgtr2* were recorded (at both time points).

Overall, clear and statistically significant changes in the liver (cell proliferation, hypertrophy and enzyme activity as well as associated changes in gene expression) were observed following Fluopyram treatment. These changes were recorded as early as following 3 days of treatment and starting from 150 ppm. The dose of 75 ppm was considered as a No Observed Adverse Effect Level (NOAEL; based on the increased gene expression of *Cyp3a3* at this dose level) and 30 ppm as a No Observed Effect Level (NOEL).

[redacted] (2012); Fluopyram – Mechanistic 3-day toxicity study in the mouse by oral gavage (thyroid hormone investigations); Bayer S.A.S., Bayer CropScience; Report No.: SA 10430; Document No.: M-426994-01.

Summary: The objective of this study was to investigate the mode of action by which fluopyram gives rise to thyroid tumors in the male mouse following chronic exposure. The effects of different dose levels of fluopyram (including the dose level which induced thyroid tumors) on the plasma levels of thyroxine (T4) and thyroid stimulating hormone (TSH) were determined following daily oral gavage for 3 days. Four time points were evaluated 2h, 8h, 14h and 48h after the last gavage administration.

Fluopyram, a fungicide of the pyramide family (batch number: Mix-batch: 08528/0002: Light beige solid, 94.7% purity), was administered daily by oral gavage for 3 days to groups of 15 male C57BL/6J mice, at dose levels of 100 or 300 mg/kg body weight/day of fluopyram. T4 and TSH hormone levels were examined at four time points 2h, 8h, 14h and 48h after the last gavage administration. One group from

each dose level was sacrificed at each time point. Four similarly constituted groups of 15 males received the vehicle alone (0.5% methylcellulose) and acted as controls. Clinical signs were recorded daily and body weight was measured on Day 1. A detailed physical examination was performed once during the treatment period. On the day of sacrifice, before necropsy, blood samples were taken for hormone level measurements (T4 and TSH).

There were no mortalities and no treatment-related findings in term of clinical signs during the course of the study.

In the fluopyram treated animals, after 3 days of exposure, at 100 or300 mg/kg/day, where animals were sacrificed 2h, 8h, 14h and 48h after the last dose, mean T4 levels in the plasma were statistically significantly ($p \leq 0.01$) decreased by between -18% and -41%. The magnitude of the response occurred in a dose-related manner at all time-points apart from the 2h time point. Mean TSH levels in the plasma were not changed when compared to controls at any of the time points investigated.

In conclusion, this study demonstrates that fluopyram administration to C57BL/6J male mice by oral gavage for three days at concentrations similar to or above the top dose level administered in the mouse cancer bioassay induced a statistically significant decrease in plasma T4 levels at all the time points examined. However, no change in plasma TSH level was detected in this short term assay.

[redacted] (2012); Fluopyram – Mechanistic 28-day toxicity study in the mouse by dietary administration (hepatotoxicity and thyroid hormone investigations); Bayer S.A.S., Bayer CropScience; Report No.: SA 11105; Document No.: M-428031-01.

Summary: The objective of the present study was to investigate the mode of action by which fluopyram gives rise to thyroid tumors in the male mouse following chronic exposure. The effects of different doses of fluopyram on the plasma levels of Thyroxine (T4) and Thyroid Stimulating Hormone (TSH), the gene expression of *Tsh* in the pituitary gland and the hepatic UDP-glucuronosyltransferases (UDPGT) activity were determined following continuous dietary administration for at least 28 days. In addition, the reversibility of any effects observed at the highest dose level was assessed following a recovery period of one month on untreated control diet. Phenobarbital was administered by oral gavage and was used as a positive control for

liver and thyroid effects. The reversibility of any effects induced by phenobarbital was also assessed following a recovery period of one month on untreated control diet.

Fluopyram, a fungicide of the pyramide family (batch number: Mix-batch: 08528/0002: Beige powder, 94.7% purity), was administered to male C57BL/6J mice continuously for at least 28 days in the diet at dose levels of 0, 30, 75, 150, 600 and 750 ppm. These doses equated to 0, 5, 13, 25, 102 and 128 mg/kg/day, respectively. A group of animals dosed with 80 mg/kg/day phenobarbital (batch number: 09050075: White crystalline powder, 100% purity) by gavage acted as a positive control for the parameters investigated in this present study. Each group consisted of 15 male mice with the exception of the control group, the 750 ppm fluopyram group and the phenobarbital group, where 15 additional males were fed untreated control diet for a further one month to assess the reversibility of changes induced during the 28 day treatment period. Clinical observations were performed daily, body weight and food intake was measured weekly. A detailed physical examination was performed once during the acclimatization phase and at least weekly during the dosing period. Animals were sacrificed either following at least 28 days of treatment or at the end of the recovery phase. All animals were subjected to a necropsy and the liver was weighed. At both sacrifice times, blood samples were taken for hormone analysis (T4 and TSH) and the liver and the pituitary gland from each animal were collected. The pituitary gland was flash frozen in liquid nitrogen and stored at approximately -74°C + 10°C until used for *Tsh* transcript analyses by quantitative Polymerase Chain Reaction (qPCR). At both sacrifice times, 5 pools of 3 livers per group were homogenized for microsomal preparations in order to determine UDPGT specific enzyme activities using either bilirubin or T4 as substrate.

Dosing phase:

Fluopyram:

There were no mortalities or treatment-related clinical signs. There were no changes in body weight parameters or food consumption during the course of the study. Fluopyram had no effect on terminal body weight but induced treatment-related changes in the liver and thyroid hormonal parameters investigated as follows: At 30 ppm, a statistically significant decrease (-28%; $p<0.01$) in mean T4 levels was recorded at terminal sacrifice.

At 75 ppm, a statistically significant decrease (-31%; p<0.01) in mean T4 levels was recorded at terminal sacrifice. At necropsy, a slight but statistically significant increase in mean liver weight relative to body weight (+6%; p<0.05) was recorded.

At 150 ppm, a statistically significant decrease (-25%; p<0.01) in mean T4 levels was recorded at terminal sacrifice. At necropsy, statistically significant increases in absolute mean liver weight and mean liver weight relative to body weight (+11%; p<0.01; +9%; p<0.01 respectively) were recorded. Hepatic UDPGT enzymatic activities were marginally, though not statistically significantly, increased (+32% for bilirubin and +52% for T4 substrates), compared to the controls.

At 600 ppm, a statistically significant decrease (-37%; p<0.01) in mean T4 levels was recorded at terminal sacrifice. At necropsy, statistically significant increases in absolute mean liver weight and mean liver weight relative to body weight (+27%; p<0.01 for both parameters) were recorded. Statistically significant increases in hepatic UDPGT enzymatic activities were observed (+39%; p<0.05 for bilirubin and +83%; p<0.01 for T4 substrates), compared to controls. In addition, an increase in the level of accumulation of *Tsh* transcript in the pituitary gland was recorded (+43%; p<0.05).

At 750 ppm, a statistically significant decrease (-38%; p<0.01) in mean T4 levels was recorded at terminal sacrifice. At necropsy, statistically significant increases in absolute mean liver weight and mean liver weight relative to body weight (+36%; p<0.01; +33%; p<0.01 respectively) were recorded. A statistically significant increase in the hepatic UDPGT-bilirubin enzymatic activity (+48%; p<0.01) together with a marginal increase of the UDPGT-T4 enzymatic (+33%) activity were observed. In addition, an increase in the level of accumulation of *Tsh* transcript in the pituitary gland was recorded (+54%; p<0.01).

Phenobarbital:

One animal treated with phenobarbital was killed for humane reasons and two others were found dead during the first week of treatment. The premature deaths of these animals may be due to the use of a concentrated phenobarbital formulation (16 g/l at a dosing volume of 5 ml /kg body weight) being administered during the first week of treatment. All males dosed with phenobarbital displayed reduced motor activity. Body weight and body weight gain were reduced throughout treatment, resulting in a statistically significant 5% (p<0.01) reduction in body weight and a statistically significant 42% (p<0.01) reduction in cumulative body weight gain by Day 29. A

statistically significant decrease (-23%; p<0.01) in mean T4 levels was recorded at terminal sacrifice. At necropsy, statistically significant increases in absolute mean liver weight or mean liver weight relative to body weight (+10%; p<0.01; +16%; p<0.01 respectively) were recorded. Non-statistically significant increases in the hepatic UDPGT-bilirubin enzymatic (+42%) and UDPGTT4 enzymatic (+32%) activities were observed. In addition, an increase in the level of accumulation of Tsh transcript in the pituitary gland was recorded (+53%; p<0.01).

Recovery phase:

Fluopyram:

In the males initially treated with 750 ppm fluopyram, a slight increase in the level of accumulation of *Tsh* transcript in the pituitary gland was recorded (+12%; p<0.05) however, the observed increase was much lower than the one observed immediately following treatment with 750 ppm (+54%; p<0.01). All other parameters were comparable with the control group.

Phenobarbital:

In the males initially treated with 80 mg/kg/day phenobarbital, body weight was 5% lower than the control group at the start of the recovery phase, the effect being statistically significant (p<0.05). By the end of the recovery phase, body weight was marginally reduced by 3% the effect was not statistically significant. All other parameters were comparable with the control group.

In conclusion, this study demonstrates that fluopyram administration at the tumorigenic dose level of 750 ppm for at least 28 days in the C57BL/6J mouse, induced a decrease in plasma T4 levels correlated with an increase in the levels of *Tsh* transcript (beta subunit) in the pituitary gland, together with an increase in the UDPGT-bilirubin and UDPGT-T4 enzymatic activities. In addition, absolute and relative liver weights were significantly increased. These effects were not detected after a four-week recovery phase except for a marginal increase in *Tsh* transcript level. All these effects occurred in a dose-related manner, apart from the decrease in plasma T4 for which there was no dose-related concordance in terms of the magnitude of the response observed. At the low dose of 30 ppm, the only finding was a decrease in plasma T4 levels, the relevance of which is doubtful based on the lack of clear dose response concordance.

[redacted] (2012); Fluopyram - Mechanistic investigations in the liver of female rats following dietary administration; Bayer S.A.S., Bayer CropScience; Report No.: SA 11104; Document No.: M-427431-01.

Summary: The objective of the present study was to investigate the mode of action by which fluopyram gives rise to liver tumors in the female rat following chronic exposure. The effects of different doses of fluopyram on the liver were determined following continuous dietary administration for at least 28 days. In addition, the reversibility of any effects observed at the highest dose level was assessed following a recovery period of one month on untreated control diet. Liver cell proliferation, hepatic total cytochrome P-450 as well as enzymatic activities and gene expression profiles for inducible P-450 and UDPGT enzymes were assessed. Liver histopathology was also evaluated. The CAR/PXR nuclear receptor activator, phenobarbital, was administered by oral gavage and was used as a positive control for the liver effects. The reversibility of any effects induced by phenobarbital was also assessed following a recovery period of one month on untreated control diet. Consequently, groups of adult female Wistar rats were exposed to fluopyram for at least 28 days in the diet at dose levels of 0, 30, 75, 150, 600 and 1500 ppm, which equated to 2.2, 5.6, 11.3, 44.5 and 111.4 mg/kg/day respectively. A group of animals dosed with 80 mg/kg/day phenobarbital by gavage acted as a positive control for the parameters investigated in this present study. Each group consisted of 15 females, apart from the negative control and 1500 ppm fluopyram group, together with phenobarbital group, where an additional 15 animals per group following the 28 days of treatment were maintained for a 1 month recovery period on untreated control diet.

Treatment Phase:

There were no relevant changes in any of the body weight parameters during the treatment phase for either fluopyram or phenobarbital. Fluopyram treatment caused ocular discharge and red conjunctiva in some females of Groups 3 (75 ppm), 4 (150 ppm) and 5 (600 ppm). In addition, food consumption was statistically significantly reduced for females treated with 1500 ppm fluopyram during week 3 ($p \leq 0.05$) and week 4 ($p \leq 0.01$) of treatment. All females dosed with phenobarbital displayed reduced motor activity and some females had ocular discharge and/or lacrimation in either one or both eyes. Other clinical signs included increased salivation and soiling around the mouth or the head. Food consumption was increased during treatment with phenobarbital ($p \leq 0.05$ for weeks 1 and 4; $p \leq 0.01$ for week 2). Fluopyram had no effect on terminal body weight but induced treatment-related changes in all liver

parameters investigated. The number of parameters affected and also the magnitude of the responses were dose-related as described below.

At 30 ppm, a statistically significant ($p \leq 0.01$) increase in the expression of *Cyp3a3* was recorded (+81%), which increased with increasing dose up to +4943% at 1500 ppm. This increase in gene expression was, however, not associated with any statistically significant increase in corresponding enzyme (BROD) activity until 600 ppm.

At 75 ppm, enlarged liver was observed in 3/15 females and centrilobular cell proliferation was marginally, but statistically significantly ($p \leq 0.05$), increased (+47%) compared to the controls.

At 150 ppm, slight but statistically significant increases in mean liver weight relative to body weight (+7%; $p \leq 0.01$) and brain weight (+11%; $p \leq 0.05$) were recorded. This increased liver weight could be associated with enlarged liver in 5/15 females. Centrilobular and global hepatic cell proliferation (+106% and +70% respectively) were statistically significantly ($p \leq 0.01$) increased compared to the controls. In addition, statistically significant ($p \leq 0.01$) increases were recorded for the expression of the Phase I genes *Cyp1a1* (+711%), *Cyp2b1* (+990%) and for the Phase II genes *Gstm4* (+136%) and *Udpgtr2* (+64%). Statistically significantly ($p \leq 0.01$) increased hepatic enzyme activity was, however, only observed for UDPGT-bilirubin (corresponding to *Udpgtr2*) and EROD (corresponding to *Cyp1a1*) starting from this dose level.

At 600 ppm, marked changes to the liver were observed as evidenced by statistically significantly increased absolute and relative liver weights (+10 to +15%), coupled with enlarged liver in 4/15 animals and minimal hepatocellular hypertrophy in 6/15 females. However, despite these macroscopic and microscopic changes, hepatic cell proliferation was similar to that observed at 150 ppm with centrilobular and global proliferation being statistically significantly increased (+106% and +67% respectively; $p \leq 0.01$). Genes coding for additional Phase II enzymes were statistically significantly increased at this dose level (*Gsta2*: +53% and *Ephx1*: +116%). A marginal, though statistically significant (+75%; $p \leq 0.01$), increased expression of the marker for cell proliferation (*Gadd 45b*) was also observed. Increased activity of BROD ($p \leq 0.01$), PROD ($p \leq 0.05$) and UDPGT-4-nitrophenol ($p \leq 0.01$) were recorded from this dose level.

At 1500 ppm, the hepatic effects were more marked than those recorded at the lower doses. Specifically, for cell proliferation the % increase (compared to controls) in global cell proliferation was 188% compared to 67% at 600 ppm. Statistically significant increases in mean absolute and relative liver weight (+29 to +33%) were also recorded at this dose level as well as enlarged liver in 14/15 animals, which were associated with the centrilobular to panlobular hypertrophy observed in 14/15 females. Changes in gene expression and enzyme activity observed at the lower doses were also more marked at this top dose level.

Phenobarbital was used as a positive control for the various parameters measured in the present study and as such induced changes in the liver pertinent for a compound that activates CAR/PXR nuclear receptors. Liver weight (absolute and relative to brain and body weight) was statistically significantly increased following phenobarbital treatment and enlarged liver was noted in 10/15 animals, which could be associated with the centrilobular to panlobular hypertrophy observed in 12/15 females and an increased number of mitoses was observed in 1/15 females. Increased cell proliferation was observed with the effects being more apparent in the centrilobular region (+335% compared to controls) than in the perilobular region (+23% compared to controls). Furthermore, the gene expression of *Gadd45b* was statistically significantly increased (+68%; $p \leq 0.01$). Statistically significant ($p \leq 0.01$) increases in the gene expression of *Cyp2b1*, *Cyp3a3* and *Udpgtr2* were recorded, which could be associated with the statistically significantly increased hepatic enzyme activity of PROD (+8.44-fold; $p \leq 0.01$), BROD (+88-fold; $p \leq 0.01$) and UDPGT-bilirubin (+1.38-fold; $p \leq 0.05$) respectively. Enzyme activity of the second isoform of UDPGT (UDPGT-4-nitrophenol) was also statistically significantly increased (+1.81-fold; $p \leq 0.01$) and increased expression of genes coding for additional Phase II enzymes (*Gstm4*, *Gsta2* and *Ephx1*) was also recorded following phenobarbital treatment.

Recovery Phase:

For the females previously treated with 1500 ppm fluopyram, there were no relevant changes in any of the body weight parameters during the recovery phase. A statistically significant reduction ($p \leq 0.05$) in food consumption was, however, recorded during the second and third week of the recovery phase. There was no effect on terminal body weight or any organ weights at the end of the recovery phase nor were there any macroscopic or microscopic changes recorded in the liver. Centrilobular and global hepatic cell proliferation were still statistically significantly

increased compared to the controls (+81%, $p \leq 0.01$ and +51%, $p \leq 0.05$, respectively); however these observed increases were lower than those observed immediately following treatment with 1500 ppm fluopyram (215% and 188% for centrilobular and global cell proliferation, respectively). Hepatic molecular and enzymatic changes were still apparent at the end of the recovery phase as evidenced by increased enzyme activity/gene expression for BROD/*Cyp3a3*, PROD/*Cyp2b1*, EROD/*Cyp1a1* and UDPGT-bilirubin. Marginal increases in the expression of *Gstm4*, *Ephx2* and *Gadd45b* were also recorded. As with the cell proliferation parameters, the magnitude of these molecular and enzymatic changes was much lower than those recorded immediately following treatment.

A similar profile was observed for the females previously treated with 80 mg/kg/day phenobarbital. In these animals a loss or a reduction in body weight gain was recorded during the first two weeks of the recovery phase, but there was no effect on terminal body weight or any organ weights at the end of the recovery phase nor were there any macroscopic or microscopic changes recorded in the liver. Hepatic cell proliferation was still statistically significantly increased compared to the controls; however the magnitude of the increases were generally lower than those observed immediately following treatment with phenobarbital. Hepatic molecular and enzymatic changes were still apparent at the end of the recovery phase as evidenced by increased enzyme activity and/or gene expression for, PROD/*Cyp2b1*, EROD, *Cyp3a3* and UDPGT-bilirubin. Marginal increases in the expression of *Cyp4a1*, *Gstm4* and *Ephx1* were also recorded. As with the cell proliferation parameters, the magnitude of these molecular and enzymatic changes was generally lower than those recorded immediately following treatment.

Overall, treatment with fluopyram for at least 28 days induced clear and statistically significant changes in the liver (cell proliferation, hypertrophy and enzyme activity as well as associated changes in gene expression). These changes were dose-related beginning from 75 ppm. The dose of 30 ppm was considered as a No Observed Adverse Effect Level (NOAEL; based on the increased gene expression of *Cyp3a3*, with no other correlated findings, at this dose level). The hepatic changes appeared to be reversible as evidenced by the reduced hepatic responses recorded in females previously treated with 1500 ppm fluopyram following the recovery period.

[redacted] (2012); Fluopyram – 28-day toxicity study for proliferation assessment in the C57BL/6J male mouse; Bayer S.A.S., Bayer CropScience; Report No.: SA 11123; Document No.: M-428303-01.

Summary: The objective of the present study was to investigate possible thyroid cell proliferation, caused by fluopyram administration to C57BL/6J male mice following continuous dietary administration for at least 28 days at a dose level (750 ppm) which caused an increase in thyroid follicular cell adenomas following chronic exposure. Fluopyram, (batch number: Mix-batch: 08528/0002: a beige powder, 94.7% w/w), a fungicide of the pyramide family, was administered daily in the diet to groups of adult male C57BL/6J mice (15 mice/dose level) for at least 28 days at a dose level of 750 ppm. This dose equated to 127 mg/kg/day. A group of animals dosed by oral gavage with 80 mg/kg/day phenobarbital (a reference compound known to induce thyroid changes in rodents, batch number: 09050075; a white crystalline powder, 100% purity) acted as a positive control for thyroid effects since it was used in a previous mouse mechanistic study, where thyroid hormone level changes were detected. A control group received untreated diet. All animals were exposed to BrdU in their drinking water during the last week of the study. Animals were observed daily for clinical signs and twice daily for moribundity and mortality except on weekends when they were checked once daily. Physical examinations were performed weekly. Body weight and food consumption were recorded once weekly. All animals were necropsied in the morning following twenty eight days of treatment; the thyroid and the liver were sampled and fixed. The thyroid was examined microscopically. Additional thyroid slides were stained for BrdU cell proliferation determinations. The duodenum was also sampled and used as a positive control tissue for the cell proliferation investigations.

Fluopyram at 750 ppm in the diet:

There were no clinical signs and no effects on food consumption or body weight parameters for the group treated with fluopyram. At necropsy, there was no change in mean terminal body weight when compared to control animals. Enlarged and dark livers were found in 14/15 and 5/15 animals, respectively. No microscopic changes in the thyroid gland were noted. Assessment of cell proliferation in the thyroid revealed a 1.7 fold increase ($p \leq 0.01$) in BrdU labeling index when compared to the controls.

Phenobarbital (reference item) at 80 mg/kg/day by gavage:

Clinical signs consisting of reduced motor activity were recorded for all mice dosed with phenobarbital from Study Day 2 onwards. In addition, mean body weight was reduced by between 3.5% ($p \leq 0.05$) and 4.8% ($p \leq 0.01$), throughout the study. Following an initial body weight loss between Study Days 1 and 8, overall cumulative body weight gain was reduced by 52% ($p \leq 0.01$) by Study Day 29, compared to the controls.

At necropsy, mean terminal body weight was lower (-4.4%, $p \leq 0.01$) when compared to control animals. Enlarged and dark livers were found in 3/15 and 4/15 animals, respectively. No microscopic changes in the thyroid gland were noted. Assessment of cell proliferation in the thyroid revealed no change in the BrdU labeling index when compared to the controls. These results are consistent with the literature, which show that phenobarbital induces thyroid cell proliferation in mice at a dose level of 1800 ppm, which corresponds approximately to 200 mg/kg/day.

In conclusion, a clear increase in thyroid cell proliferation, as evidenced by a 1.7 fold increase ($p \leq 0.01$) in BrdU labeling index, compared to the controls, was observed following dietary administration of fluopyram at 750 ppm for at least 28 days, to the male C57BL/6J mouse.

[redacted] (2013). Fluopyram: 28-day dietary mouse study (assess thyroid cell proliferation, with reversibility). Bayer S.A.S., Bayer CropScience; Study Completion Date: 2013-03-27, Edition Number: M-449821-01-1; Study Report Number: SA 12058.

The objective of the present study was to characterize the thyroid effects caused by the administration of different dose levels of fluopyram to C57BL/6J male mice following continuous dietary administration for at least 28 days and to determine a threshold for thyroid follicular cell proliferation. In addition, the reversibility of any effects observed at the high dose level was assessed following a recovery period of at least 28 days on untreated control diet. Fluopyram, (batch number: Mix-batch: 08528/0002: a beige powder, 94.7% w/w purity), a fungicide of the pyridinyl-ethylbenzamide (pyramide) family, was administered daily in the diet to groups of adult male C57BL/6J mice for at least 28 days at dose levels of 0, 30, 75, 150, 600, 750 and 1500 ppm. These doses equated to 0, 5, 13, 25, 99, 124 and 247 mg/kg/day, respectively. Each group consisted of 15 male mice with the exception of the control group and the 1500 ppm fluopyram group, where 15 additional males were fed

untreated control diet for a further one month to assess the reversibility of changes induced during the 28 day treatment period. All animals were exposed to BrdU in their drinking water during the last week of the study. Animals were observed daily for clinical signs and twice daily for moribundity and mortality except on weekends when they were checked once daily. Physical examinations were performed weekly. Body weight and food consumption were recorded once weekly. All animals were sacrificed either following at least 28 days of treatment or at the end of the recovery phase. All surviving animals were subjected to necropsy and the thyroid and the duodenum were sampled and fixed. The thyroid was examined microscopically. Additional thyroid slides were stained for BrdU cell proliferation determinations. The duodenum was also sampled and used as a positive control tissue for the cell proliferation investigations.

Dosing phase

No treatment-related clinical signs, effects on body weight parameters, food or water consumption were observed at any dose level tested. At necropsy, there was no change in mean terminal body weight when compared to control animals at any dose level tested. No treatment-related macroscopic and microscopic changes in the thyroid gland were noted. Assessment of cell proliferation in the thyroid revealed a statistically significant 1.43 and 2.07 fold increase ($p<0.01$ and $p<0.001$, respectively) in BrdU labeling index at 750 and 1500 ppm, respectively, when compared to the controls.

Recovery phase

In group initially dosed at 1500 ppm, there were no treatment-related clinical signs and no effects on body weight parameters, food or water consumption.

At necropsy, there was no change in mean terminal body weight when compared to control animals. No macroscopic changes in the thyroid gland were noted. Assessment of cell proliferation in the thyroid revealed no change in BrdU labeling index in the 1500 ppm dose group when compared to the controls.

In conclusion, a clear increase in thyroid cell proliferation, as evidenced by a 1.43 and 2.07 fold increase ($p<0.01$ and $p<0.001$, respectively) in BrdU labeling index, compared to the controls, was observed following dietary administration of fluopyram at 750 and 1500 ppm, respectively, for at least 28 days, to the male C57BL/6J mouse, this phenomenon being reversible (no more cell proliferation observed after a 28 day wash out period).

[redacted] (2013). Fluopyram: 28-day Wild Type versus CAR / PXR Knock Out mouse study. Bayer S.A.S., Bayer CropScience; Study Completion Date: 2013-03-27; Edition Number: M-449890-01-1; Study Report Number: SA 12162.

Fluopyram (batch number: Mix-batch: 08528/0002, a beige powder, 94.7% purity), a fungicide of the pyridinyl-ethylbenzamide family, was administered continuously via the diet to groups of C57BL/6J or Pxr KO/Car KO mice (15/group/strain) for at least 28 days at concentrations of 0, 750 and 1500 ppm, corresponding to 125 and 256 mg/kg/day in C57BL/6J male mice and to 130 and 247 mg/kg/day in Pxr KO/Car KO male mice. A similarly constituted group received untreated diet and acted as a control. All animals were exposed to BrdU in their drinking water during the last week of the study. Animals were observed at least once daily for clinical signs and twice daily for moribundity and mortality except on weekends when they were checked once daily. A detailed physical examination was performed weekly throughout the study. Body weight and food consumption were recorded once weekly. Before necropsy a blood sample was collected from the abdominal aorta of each animal for possible further analysis. All animals were necropsied, brain and liver were weighed. Liver, thyroid gland, duodenum and pituitary gland were sampled. Thyroid gland, duodenum and two central sections taken from the median and left lobes of the liver were fixed and examined microscopically. Additional thyroid slides were stained for BrdU cell proliferation determinations. The duodenum was also sampled and used as a positive control tissue for the cell proliferation investigations. The pituitary gland was collected and flash frozen in liquid nitrogen and stored at approximately -74°C + 10°C for gene transcript analyses by quantitative Polymerase Chain Reaction (q-PCR). In addition, one piece of both the median and the left lobe of the liver from each animal were collected and flash frozen in liquid nitrogen and stored at approximately -74°C + 10°C for possible gene transcript analyses. The remaining portions of the liver from all animals were collected and flash frozen in liquid nitrogen prior to storage at approximately -70°C until shipped to CXR Bioscences for microsomal preparations in order to determine total cytochrome P-450 content, cytochrome P-450 isoenzyme and UDPGT specific enzyme activities.

C57BL/6J mice

No clinical signs were observed throughout the study. Body weight, food consumption and water consumption parameters were unaffected by treatment throughout the study when compared to the controls.

At 1500 ppm. At necropsy, mean absolute and relative liver weights were statistically significantly higher when compared to controls. Enlarged livers were noted in 14/15 animals, compared with 0/15 animals in the control group. Microscopic examination revealed hepatocellular hypertrophy, hepatocellular single cell necrosis, increased number of mitoses and interstitial mixed cell infiltrate. Assessment of cell proliferation in the thyroid revealed a 2.6 fold ($P \leq 0.001$) higher BrdU labeling index, when compared to the controls. Regarding the hepatotoxicity testing, total cytochrome P-450 content was increased (3.7 fold, $P \leq 0.001$) as were PROD (150 fold, $P \leq 0.001$), BQ (7.9 fold, $P \leq 0.001$), T_4-GT (1.9 fold, $P \leq 0.001$) and BIL-GT (2.0 fold, $P \leq 0.001$) activities. In the pituitary gland, *Tshb* gene transcripts were up-regulated (1.7 fold, $P \leq 0.001$) when compared to the controls.

At 750 ppm. At necropsy, mean absolute and relative liver weights were statistically significantly higher (+39% to +42%, $P \leq 0.01$) when compared to controls. Enlarged livers were noted in 7/15 animals, compared with 0/15 animals in the control group. Microscopic examination revealed hepatocellular hypertrophy, hepatocellular single cell necrosis and interstitial mixed cell infiltrate. Assessment of cell proliferation in the thyroid revealed a 1.8 fold ($P \leq 0.001$) higher BrdU labeling index, when compared to the controls. Regarding the hepatotoxicity testing, total cytochrome P-450 content was increased (3.6 fold, $P \leq 0.001$) as were PROD (70 fold, $P \leq 0.001$), BQ (5.5 fold, $P \leq 0.001$), T_4-GT (1.8 fold, $P \leq 0.001$) and BIL-GT (1.8 fold, $P \leq 0.001$) activities. In the pituitary gland, *Tshb* gene transcripts were up-regulated (1.6 fold, $P \leq 0.01$) when compared to the controls.

Pxr KO/Car KO mice

No treatment-related clinical signs were observed throughout the study. Body weight, food consumption and water consumption parameters were unaffected by treatment throughout the study when compared to the controls.

At 1500 ppm. At necropsy, mean absolute and relative liver weights were statistically significantly higher when compared to controls. Regarding the hepatotoxicity testing, PROD activity was slightly induced (1.4 fold, $P \leq 0.01$) whereas BQ and T_4-GT activities were decreased (1.7 fold, $P \leq 0.001$ and 1.3 fold, $P \leq 0.01$;

respectively). In the pituitary gland, *Tshb* gene transcripts were slightly down-regulated (1.2 fold, P≤0.05) when compared to the controls.

At 750 ppm. At necropsy, mean absolute and relative liver weights were statistically significantly higher when compared to controls. Regarding the hepatotoxicity testing, PROD activity was slightly induced (1.4 fold, P≤0.001) whereas BQ activity was decreased (1.5, P≤0.001). No were no changes in *Tshb* gene transcripts.

Conclusion:

The clear increases in PROD activity seen in wild type C57BL/6J mice, with minimal PROD induction in Pxr KO/Car KO mice, after exposure to fluopyram, indicates that fluopyram is an inducer of Cyp2b in wild type mice. The induction of BQ activity in wild type mice and decrease in BQ activity in Pxr KO/Car KO mice shows that fluopyram is also a Cyp3a inducer in WT mice.

These data, together with the increases in the glucuronidation of bilirubin and thyroxine seen in WT mice but not in Pxr KO/Car KO mice after exposure to fluopyram, clearly demonstrate that fluopyram is a Car and Pxr activator in mice. This is translated as in an increase in *Tshb* gene transcripts and in an increase of thyroid follicular cell proliferation.

[redacted] (2013). Fluopyram: Comparative assessment of enzyme and DNA synthesis induction in cultured rat hepatocytes. Study Completion Date: 2013-04-02; Edition Number: M-450157-01-1; Study Report Number: CXR1242.

Female rat hepatocytes were cultured in the presence of the test item fluopyram, and phenobarbital or EGF as two positive control compounds. A decrease in ATP concentration at 300 µM fluopyram was observed. This was indicative of cytotoxicity. Fluopyram was not cytotoxic at 100 µM. Culturing primary female rat hepatocytes with fluopyram stimulated replicative DNA synthesis in a dose-dependent manner. At 100 µM, S-phase was increased by approximately 3.9–fold. Similar, but smaller, increases were observed following exposure of hepatocytes to phenobarbital (2.9–fold at 100 µM). Culturing primary rat hepatocytes with fluopyram resulted in an increase in pentoxyresorufin-O-depentylation (PROD) activity of up to a maximum of 2.8–fold at 30 µM. This indicates that fluopyram is an inducer of CYP2B. Culturing primary rat hepatocytes with fluopyram resulted in an increase in benzyloxyresorufin-O-debenzylation (BROD) activity, up to a maximum

of 4.2–fold at 30 µM. This indicates that fluopyram is an inducer of CYP2B/CYP3A. Culturing primary rat hepatocytes with fluopyram resulted in a concentration-dependent increase in benzyloxyquinoline-O-debenzylation (BQ) activity up to a maximum of 18.0–fold at 100 µM. This indicates that fluopyram is an inducer of CYP3A. In conclusion, these data suggest that fluopyram is an activator of both CAR and PXR.

[redacted] (2013). Fluopyram: Enzyme and DNA-synthesis induction in cultured human hepatocytes, main study. Study Completion Date: 2013-04-02; Edition Number: M-450156-01-1; Study Report Number: CXR1241.

Female human hepatocytes were cultured in the presence of the test item fluopyram, and phenobarbital or EGF as two positive control compounds. A decrease in ATP concentration, commencing with a slight decrease at 100 µM followed by a marked decrease at 300 µM fluopyram, was observed. This was indicative of cytotoxicity. Fluopyram was not cytotoxic at 30 µM. Culturing primary female human hepatocytes with fluopyram had no effect on replicative DNA synthesis. Likewise no effect was observed following exposure of hepatocytes to phenobarbital. Culturing primary human hepatocytes with fluopyram resulted in an increase in pentoxyresorufin-O-depentylation (PROD) activity of up to a maximum of 1.9–fold at 100 µM. This indicates that fluopyram is an inducer of CYP2B. Culturing primary human hepatocytes with fluopyram resulted in an increase in benzyloxyresorufin-O-debenzylation (BROD) activity, up to a maximum 2–fold at 300 µM. This indicates that fluopyram is an inducer of CYP2B/CYP3A. Culturing primary human hepatocytes with fluopyram resulted in an increase in benzyloxyquinoline-O-debenzylation (BQ) activity up to a maximum of 1.8–fold at 10 µM. This indicates that fluopyram is an inducer of CYP3A at low concentrations. In conclusion, these data suggest that fluopyram is a weak activator of CAR and PXR, with no compound-stimulated DNA synthesis in human hepatocytes.

[redacted] (2013). Fluopyram: Assessment of pentoxyresorufin-*o*-depentylation and benzyloxyquinoline-*o*-debenzylation in 50 liver microsomal samples. Study Completion Date: 2013-; Edition Number: M-451628-01-1; Study Report Number: CXR1284.

Microsome samples (50 samples) were received, frozen, by CXR on 03-Aug-2011 and stored at approximately -70 °C prior to analysis. Microsome preparation and measurement of total protein concentration was carried out by Bayer S.A.S (Study number SA 11105)[1].

The activity of Cyp2b was measured in the mouse liver microsome preparations as the rate of depentylation of pentoxyresorufin (PROD).

28 days dietary administration of fluopyram resulted in a dose-dependent increase in PROD activity to a maximum 47.2-fold at 750ppm. 28 days administration of phenobarbital at 80mg/kg/day resulted in a 32.7-fold increase in PROD activity.

Following a 28 day recovery period on control diet, PROD activity in both fluopyram and phenobarbital treated mice returned to control levels.

The activity of Cyp3a was measured in the mouse liver microsome preparations as the rate of debenzylation of benzyloxyquinoline (BQ).

28 days dietary administration of fluopyram resulted in a dose-dependent increase in BQ activity to a maximum 6.2-fold at 750ppm. 28 days administration of phenobarbital at 80mg/kg/day resulted in a 3.0-fold increase in BQ activity.

Following a 28 day recovery period on control diet, BQ activity in both fluopyram and phenobarbital treated mice returned to control levels.