Bayer CropScience



Document Title

# Tier 2 Summary of the Toxicological and Toxicokinetic Studies on the Active Substance for

# Fluopyram (AE C656948)

## Extract Covering Mechanistic Data

Data Requirements

**EU Directive 91/414 EEC**
**Regulatory Directive 2003-01/Canada/PMRA**
**OPPTS guidelines/US/EPA**

**Annex IIA**
**Section 3, Point 5**
**Document M**

**According to OECD format guidance for industry data submissions on plant protection products and their active substances**

Date

**2013-12-02**

Authors

[redacted]

**Bayer CropScience**

## IIA 5.5.4 Mechanism of action and supporting data

**Supporting data regarding female rat liver tumors**

In the rat carcinogenicity study (see 5.5.2), a higher incidence of liver cell tumors (hepatocellular adenoma + carcinoma) was observed in females after 2-years of treatment with AE C656948 at 1500 ppm (89 mg/kg bw/d). No neoplastic changes were observed in males (highest dose = 29 mg/kg bw/d).

This neoplastic change was observed together with a number of proliferative changes (including altered hepatocytes (eosinophilic foci), clear cell foci, multinucleated hepatocytes with anisocaryosis or increased number of mitoses) in association with metabolic morphological changes (centrilobular to panlobular hypertrophy, focal/multifocal hepatocellular vacuolation, centrilobular to midzonal hepatocellular macrovacuolation, brown pigment in Kupffer cells or hepatocellular brown pigment).

In the 28-day study in the rat by oral administration (see 5.3.1), hepatotoxicity testing showed that AE C656948 was able to induce total cytochrome P-450 together with BROD and PROD activities and therefore was considered to be a moderate phenobarbital-like cytochrome P-450 inducer. Consequently, a "phenobarbital-like" mode of action (MoA) was anticipated for liver cell tumor formation, especially as phenobarbital is known to stimulate cell proliferation in normal hepatocytes in rodents resulting in a liver tumor promotion. In addition to its ability to induce liver enzymes and to increase liver cell proliferation, phenobarbital responses that are key for its tumorigenic effect include among others, hypertrophy and development of altered hepatic foci (Whysner *et al*., 1996[1]) which were all effects observed in the rat carcinogenicity study with AE C656948. These data support a similar MoA for AE C656948 and phenobarbital regarding the liver tumors observed in the female rat in the carcinogenicity study. As for liver enzyme induction, these effects are mediated through an activation of nuclear receptors, in particular the constitutive androstane receptor (Car) and pregnane X receptor (Pxr). Although Car and Pxr are expressed in human hepatocytes (Moore *et al*. 2003[2]), liver cell proliferation would only occur in rodents and not in humans, this is a necessary precursory event for liver tumor formation, via this MoA. In addition, there are convincing data showing that patients receiving phenobarbital for many years do not show evidence of tumorigenic effects (IARC, 2001[3]). For these reasons, the mode of action for phenobarbital-like P-450 induction is considered not to be relevant to humans (Holsapple *et al*., 2006[4]).

In order to test this hypothesis, an intial explanatory study was conducted with AE C656948 in female rats at a single high dose level of AE C656948, which was twice the high dose level used in the rat carcinogenicity study, administered for 7 days to assess its ability to cause liver cell proliferation under in-house experimental conditions. For comparison, a similar study was conducted with phenobarbital. Two further explanatory studies were subsequently conducted in the female rat to further characterize the profile of AE C656948 and to establish a dose and time concordance for activation of the key precursory events i.e. Car/Pxr nuclear receptor activation, leading to liver cell proliferation, which in the female rat ultimately results in liver tumors following chronic exposure to AE C656948 at a high enough dose level. The range of dose levels tested included the dose levels used

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[1] Whysner J., Ross P.M. and Williams G.M. (1996) Phenobarbital mechanistic data and risk assessment: enzyme induction, enhanced cell proliferation and tumor promotion. Pharmacol. Ther. 71 (1/2), 153-191.
KIIA 5.5.4 /06; Whysner, J.; Ross, P. M.; Williams, G. M.; 1996; M-300847-01

[2] Moore J.T., Moore L.B., Maglish J.M. and Kliewer S.A. (2003) Functional and structural comparison of PXR and CAR, Biochem. Biophys. Acta, Feb 17, 1919 (3) 235-238.
KIIA 5.5.4 /07; Moore, J. T.; Moore, L. B.; Maglich, J. M.; Kliewer, S. A.; 2003; M-300852-01

[3] IARC (2001) (Anon.), Phenobarbital and its sodium salts in "Some thyrotropic agents - Summary of data reported and evaluation", IARC Monograph Vol 79, IARC Press Lyon-France, pp 161-288.
KIIA 5.5.4 /08; Anon.; 2001; M-300830-01

[4] Holsapple M.P., Pitot H.C., Cohen S.H., Boobis A. R., Klaunig J.E., Pastoor T., Dellarco V.L. and Dragan Y.P. (2006) Mode of action in relevance of rodent liver tumors to human cancer risk. Toxicol. Sci. 89(1), 51-56.
KIIA 5.5.4 /09; Holsapple, M.; Pitot, H. C.; Cohen, S. H.; Boobis, A. R.; Klanig, J. E.; Pastoor, T.; Dellarco, V. L.; Dragan, Y. P.; 2005; M-300828-01

in the rat carcinogenicity study, plus intermediate dose levels, which were administered to female rats for 3, 7 or 28 days. The following parameters were assessed; induction of total cytochrome P-450 content, specific cytochrome P-450 enzyme activities and UDPGT species isoenzyme profiles and corresponding gene transcript expression, which acted as markers for activation of the Car/Pxr receptor, liver cell proliferation together with histopathological changes in the liver. Reversibility of changes seen following 28 days of treatment with AE C656948 was also assessed. In both studies, phenobarbital was included as a positive control. These explanatory studies are summarized hereafter. Overall, these studies showed that AE C656948 exhibits a similar pattern of effects as phenobarbital in our experimental system in terms of liver enzyme induction and in its capability to induce liver cell proliferation compared to untreated animals. A dose and time concordance was established for each key event and changes observed after up to 28 days of treatment with fluopyram were found to be reversible following cessation of treatment for 28 days.

In addition, a pivotal mechanistic study conducted to demonstrate Car/Pxr receptor activation as the first key event was a 28-day mouse study using both the wild-type (WT) C57BL/6J mouse and a genetically modified mouse that does not have functional Car or Pxr receptors (Pxr-Car KO), this study is summarized in the section pertaining to mouse mechanistic studies. Mice were exposed to AE C656948 at the tumorigenic dose (750 ppm) in the mouse cancer bioassay and above (1500 ppm), equivalent in terms of mg/kg/d to the rat tumorigenic dose level. In this experiment, a significant induction of liver enzymes, liver enlargement and hepatocellular hypertrophy was seen in the WT mouse, but was not observed in the Pxr-Car KO mouse.

In order to demonstrate the non-relevance of rat liver tumors to humans, an *in-vitro* comparative study was conducted to examine the proliferative response in rat and human primary hepatocytes exposed to AE C656948 at a range of dose levels up to a cytotoxic dose, details of this study are summarized hereafter. In a similar manner to phenobarbital, used as a positive control, the rat cells exposed to AE C656948 showed a dose-response increase in proliferation, whereas human cells did not. Exposure to epidermal growth factor, a positive control, resulted in a marked proliferative response in both rat and human cells. This *in-vitro* experiment clearly demonstrates that one of the crucial key events for progression of liver tumors (hepatocellular proliferation) would not take place in humans.

Other plausible MoAs for liver tumor formation that are likely to be relevant to humans have effectively been excluded. Several MoAs have been identified for liver carcinogenesis in both rodents and humans (Cohen, S.M. (2010). Evaluation of Possible Carcinogenic Risk to Humans Based on Liver Tumors in Rodent Assays: The two-year bioassay is no longer necessary. Toxicol. Pathol. 38: 487-501, 2010), KIIA 5.5.4 /36; Cohen, S. M.; 2010; M-367547-01) those highlighted in bold in the list below are likely to be relevant to humans:

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

DNA reactivity is the first broad category of a MoA for hepatocellular carcinogens. A battery of *in-vitro* genotoxicity studies; the bacterial reverse mutation test (Ames test), mammalian chromosome aberration test, and a mammalian cell gene mutation test, together with an *in-vivo* mouse micronucleus assay showed that AE C656948 does not have a genotoxic potential. Thus, DNA reactivity can be excluded as a potential MoA for the induction of tumors in rats or mice.

For non-DNA-reactive rodent liver carcinogens, several MoAs have been identified that act by stimulating hepatocellular proliferation through either a receptor- or non-receptor-mediated mechanism. The MoA studies in rats and mice with AE C656948 clearly demonstrate a specific, dose-related increase in the Cyp2b/Car-associated and Cyp3a/Pxr-associated gene and enzyme activity. Furthermore, the absence of such a response in Pxr-Car-KO mice supported the specificity for AE C656948-induced activation of Car and Pxr and excluded other receptor (Pparα, AhR, ER, statins, and cytotoxicity) and non-receptor-mediated (cytotoxicity, infections, iron overload, and increased apoptosis) involvement in the key events leading to AE C656948-induced liver tumors.

Overall, the mechanistic studies together with the standard repeat dose studies, clearly demonstrate the MoA for the rat liver tumors following a life-time exposure to AE C656948 is *via* activation of the Car/Pxr nuclear receptors. Furthermore, the lack of proliferation in primary human hepatocytes exposed to AE C656948 as compared to rat hepatocytes, provide convincing evidence that the liver tumors in the rat are non-relevant to humans. Consequently, the mode of action demonstrated to be responsible for liver tumor formation in the female rat following chronic exposure to high dose levels of AE C656948 is considered not to be relevant to humans.

Further detailed argumentation regarding mode of action and relevance to humans of the rat liver tumors following exposure to fluopyram (AE C656948) are presented in the following Expert Summary documents:

Position Paper
Fluopyram: Mode of Action and Human Relevance Analysis of Rodent Liver and Thyroid Tumors, MIIA Sec 3 /03;[redacted];2013;M-465168-01

Expert Summary Report
Fluopyram: Mode of Action and Human Relevance Framework Analysis for Fluopyram-Induced Rodent Liver and Thyroid Tumors, MIIA Sec 3 /02;[redacted];2013;M-454439-01

| | |
|---|---|
| **Report:** | **KIIA 5.5.4 /01; [redacted] 2008; M-299274-01** |
| Title: | Fluopyram (AE C 656948): 7-day mechanistic study in the female Wistar rat by dietary administration |
| Report No & | SA 07323 |
| Document No | M-299274-01 |
| **Guidelines:** | **No specific guideline** |
| **Deviations:** | **none** |
| **GLP** | **Yes** |

**Executive summary**

AE C656948 (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 the BrdU administration period. All animals were subjected to necropsy. Brains and livers 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.

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: $p \leq 0.01$). This increase was associated with macroscopic findings (enlarged and dark livers) in 13/15 females compared to no incidences 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 four fold increase in mean BrdU labeling index in both the perilobular and centrilobular 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 a marked increase in mean UDPGT activity (all statistically different from the control group ($p \leq 0.01$)). These data showed that AE C656948 has the ability to induce moderately phenobarbital-inducible hepatic enzymes (total cytochrome P-450, BROD and PROD and UDPGT) as well as liver hypertrophy and cell proliferation in the liver.

# I. MATERIALS AND METHODS

## A. MATERIALS:

**1. Test Material:** AE C656948
**Description:** Light beige powder
**Lot/Batch:** Mix-Batch:08528/0002
**Purity:** 94.7 %
**CAS:** 658066-35-4
**Stability of test compound:** Stable in rodent diet for a period covering the study duration

**2. Vehicle and /or positive control:** None

**3. Test animals:**
**Species**: Rat
**Strain:** Wistar Rj: WI (IOPS HAN) – Female only
**Age:** 11 weeks approximately at start of treatment
**Weight at dosing:** 228 to 254 g
**Acclimation period:** 12 days
**Diet:** Certified rodent powdered and irradiated diet A04C-10 P1 from [redacted] ([redacted], [redacted], France), *ad libitum*
**Water:** Tap water, *ad libitum*
**Housing:** Animals were caged individually in suspended stainless steel wire mesh cages.
**Environmental conditions –**
**Temperature:** 20 – 24°C
**Humidity:** 40 – 70 %
**Air changes:** Approximately 10-15 changes per hour
**Photoperiod:** Alternating 12-hour light and dark cycles (7 am- 7 pm)

## B. STUDY DESIGN:

**1. In life dates:** Dosing period from November 19 to 26, 2007

**2. Animal assignment and treatment**

AE C656948 was administered in the diet for 7 days to a group of 15 females at the dose of 3000 ppm (193 mg/kg/day). The dose level was selected on the basis of other studies conducted with the test substance. A negative control group with the same number of animals received plain diet. Animal housing and husbandry were in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals (Public Health Service, National Institute of Health, NIH publication N°86-23, revised 1985) and "Le Guide du Journal Officiel des Communautés Européennes L358, 18 Décembre 1986, N°86/609/CEE du 24 Novembre 1986".

Control animals were fed control diet for 7 days. Test animals were fed diet containing fluopyram (AE C656948) at 3000 ppm for 7 days. A solution of BrdU at 80 mg of BrdU/100 mL of drinking water was administered to all animals during 7 days to allow liver cell proliferation evaluation.

**3. Diet preparation and analysis**

AE C656948 was incorporated into the diet by dry mixing to provide the required concentration. There was one preparation for the whole study. The stability was demonstrated during the course of the study at concentrations of 20 and 10000 ppm for a time which covered the period of usage and storage for the study. Homogeneity of test substance in diet was verified on the formulation to demonstrate adequate formulation procedures. The mean value obtained in homogeneity check was used as measured concentration.

**Table 5.5.4-1: Study design**

| Test group | Concentration in diet (ppm) | Dose per animal (study averages) | Animals assigned |
|---|---|---|---|
| | | Female (mg/kg bw/day) | Female |
| 1 | 0 | 0 | 15 |
| 2 | 3000 | 193 | 15 |

**4. Statistics**

- Body weight change parameters,
- Terminal body weight, absolute and relative organ weight parameters,

Mean and standard deviation were calculated for each group and per time period for body weight change parameters.
The F test was performed to compare the homogeneity of group variances.
If the F test was not significant ($p>0.05$), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided).
If the F test was significant ($p\leq0.05$), mean of the exposed group was compared to the mean of the control group using the modified t-test (2-sided).

- Body weight and average food consumption/day parameters
- Total cytochrome P450 content

Mean and standard deviation were calculated for each group and per time period for average food consumption/day parameters.
The F test was performed to compare the homogeneity of group variances.
If the F test was not significant ($p>0.05$), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided).

If the F test was significant ($p\leq0.05$), data were transformed using the log transformation. If the F test on log transformed data was not significant ($p>0.05$), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided) on log transformed data.

If the F test was significant ($p\leq0.05$) even after log transformation, mean of the exposed group was compared to the mean of the control group using the modified t-test (2-sided).

If one or more group variance(s) equal 0, means were compared using non-parametric procedures.

- - Enzymatic activities and cell proliferation parameter

Mean of the exposed group will be compared to the mean of the control group using the exact Mann-Whitney test (2-sided).

Group means will be compared at the 5% and 1% levels of significance.
Statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics) except for liver enzyme parameters and cell proliferation parameters which were analyzed using SAS programs version 8.2.

## C. METHODS:

### 1. Observations

The animals were observed twice daily for moribundity and mortality (once daily on weekends or public holidays). Observed clinical signs were recorded at least once daily for all animals and detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility, and duration of any clinical signs were recorded.

### 2. Body weight

Each animal was weighed on study Day 1 and 7. Additionally, diet fasted animals were weighed before scheduled necropsy.

### 3. Food consumption

The weight of food supplied and of that remaining at the end of the food consumption period was recorded weekly for all animals during the treatment period. Any food spillage was noted. From these records, the weekly mean achieved dosage intake in mg/kg/day was calculated.

### 4. Water consumption

Drinking water bottles containing BrdU were weighed on the first day of BrdU administration. Empty water bottles were weighed on the day of scheduled sacrifice (study Day 8).

### 5. Blood sampling

On the day of necropsy, blood samples were taken from all animals in all groups by puncture of the abdominal aorta. Animals were diet fasted overnight prior to bleeding and anesthetized by inhalation of Isoflurane. Blood was collected into tubes containing lithium heparin.

### 6. Sacrifice and pathology

**Necropsy procedure – Organ sampling**
On study Day 8, all animals from all groups were sacrificed by exsanguination under deep anesthesia by inhalation of Isoflurane ([redacted], [redacted] France). Animals were diet fasted overnight prior to sacrifice.
All animals were necropsied. The necropsy included the examination of the external surfaces, all orifices and all major organs, tissues and body cavities. Brain and liver were weighed fresh at scheduled sacrifice only.
Duodenum and two central sections of the liver taken in the left and medial lobes were fixed by immersion in neutral buffered 10% formalin. The remaining portions of the liver from ten females from each group were kept for microsomal preparations and determination of total cytochrome P-450 content and isoenzyme activities.

**Histotechnology - Histopathology – Cell proliferation assessment**
Duodenum and the two central sections of the liver were embedded in paraffin wax.
Histological sections, stained with hematoxylin and eosin, were prepared and examined from all the animals.
For cell proliferation assessment, an immunohistochemical staining demonstrating the

incorporation of BrdU and the determination of the labeling index were performed to assess hepatocytic cell cycling on all study animals. The immunohistochemical reaction included incubation with a monoclonal antibody raised against BrdU, amplification with a secondary biotinylated antibody and a streptavidin-horseradish peroxidase complex, detection of the complex with the chromogen diamino-benzidine (DAB) and nuclear counterstaining with hematoxylin.
The zonal labeling index, expressed as the number of BrdU-positive hepatocytes per thousand, was measured separately on random fields comprising at least 1000 centrilobular and 1000 periportal cells using an automated image analysis system. The mean labeling indexes (periportal, centrilobular and combined) and standard deviation were calculated for each zone and each group.
The immunohistochemical staining for BrdU and determination of the labeling index were performed on all animals showing sufficient BrdU incorporation (estimated by water consumption or duodenum BrdU labeling).

**7. Hepatotoxicity testing:**

At final necropsy, the remaining portions of the liver from all surviving animals were homogenized for microsomal preparations in order to determine total cytochrome P-450 content and specific cytochrome P-450 isoenzyme profile (including EROD, BROD and PROD activities) to check the hepatotoxic potential of the test substance. Phase II enzymatic activities were also determined by measuring UDP-glucuronosyltransferase (UDPGT) with 4-nitrophenol as substrate. Results were compared to those generated with well known reference compounds.

## II. RESULTS AND DISCUSSION

### A. OBSERVATIONS:

Mortality:

There was no mortality in any group during the course of the study.

Clinical signs:

There were no treatment-related clinical signs observed in any group.

### B. BODY WEIGHT AND BODY WEIGHT GAIN:

There was no relevant change in mean bodyweight in treated animals compared to controls.

### C. FOOD and WATER CONSUMPTIONS AND COMPOUND INTAKE:

There was a slight reduction in food consumption in the treated group compared to control, however this was essentially due to a single animal. No effect was observed on water consumption.
The achieved dose in the treated group was 193 mg/kg/day.

### D. SACRIFICE AND PATHOLOGY

Higher liver weights (+ 40% approximately) were observed in animals treated with AE C656948 compared to controls. This was associated with enlarged liver observed at the macroscopic examination in nearly all animals and with hepatocellular hypertrophy in all animals at the microscopic examination.

**Table 5.5.4-2 Liver changes after 7 days of treatment with AE C656948 at 3000 ppm**

| Dosage level (ppm) | 0 | 3000 |
|---|---|---|
| **Liver weight** | | |
| Absolute (g) | 5.61 ± 0.49 | 7.86 ± 0.93** (+40%) |
| Body weight-relative | 2.53 ± 0.13 | 3.63 ± 0.320** (+43%) |
| Brain-relative | 299.50 ± 30.26 | 421.3 ± 40.62** (+41%) |
| **Microscopic pathology** | | |
| Diffuse centrilobular to panlobular hepatocellular hypertrophy | 0/15 | 15/15 |
| Diffuse mainly periportal hepatocellular vacuolation | 11/15 | 1/15 |

**: p≤0.01

**Cell proliferation**

Cell proliferation was assessed separately in the centrilobular and the periportal zones of the hepatic lobules. In the centrilobular and periportal areas, the mean BrdU labeling indexes were found to be approximately 4 times higher in treated animals (p≤0.01), when compared to controls.

**Table 5.5.4-3 Mean BrdU labeling index in the liver after 7 days of treatment with AE C656948 at 3000 ppm**

| Dosage level (ppm) | 0 | 3000 |
|---|---|---|
| Number of animals | 14 | 14 |
| BrdU positive cells in the centrilobular zone | 44.54 ± 22.31 | 179.68 ± 95.18** |
| BrdU positive cells in the periportal zone | 28.55 ± 16.80 | 112.94 ± 58.19** |
| **Overall BrdU positive cells** | **36.54 ± 18.70** | **146.31 ± 70.26**** |

**: p≤0.01

**Hepatotoxicity testing**

Changes that were observed are described in Table 5.5.4-4. 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 a marked increase in mean UDPGT activity.

**Table 5.5.4-4 Results of the hepatotoxicity testing after 7 day of treatment with AE C656948 at 3000 ppm**

| Dosage level (ppm) | 0 | 3000 |
|---|---|---|
| Number of animals | 10 | 10 |
| Total P-450 (nmol/mg prot.) | 0.91 ± 0.17 | 1.23 ± 0.20 ** (+35 %) |
| EROD (pmol/min/mg prot.) | 47.99 ± 3.73 | 103.18 ± 13.74 ** (+115 %) |
| PROD (pmol/min prot.) | 6.65 ± 0.70 | 28.55 ± 14.12** (+329 %) |
| BROD (pmol/min/mg prot.) | 6.39± 1.12 | 74.51 ± 50.89 ** (+1066 %) |
| UDPGT (nmol/min /mg prot.) | 6.42 ± 0.61 | 30.69 ± 1.94 ** (+378 %) |

**: p≤0.01
(%) as compared to control

## III. CONCLUSION

These data showed that AE C656948 has the ability to induce moderately phenobarbital-inducible hepatic enzymes (total cytochrome P-450, BROD and PROD and UDPGT) as well as liver hypertrophy and cell proliferation in the liver.

| | |
|---|---|
| **Report:** | **KIIA 5.5.4 /02; [redacted] 2008; M-299491-01** |
| Title: | Phenobarbital 7-day mechanistic study in the female Wistar rat by gavage |
| Report No & | SA 07325 |
| Document No | M-299491-01 |
| **Guidelines:** | **No specific guideline** |
| **Deviations:** | **none** |
| **GLP** | **yes** |

**Executive summary**

Phenobarbital (batch: 06100228, white crystalline powder, purity 99.6%), was administered once daily by oral gavage to groups of female Wistar rats (15/group) for 7 days at a 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 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.

Administration of phenobarbital at 80 mg/kg/day for 7 days caused reduced activity in all animals tested. 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: $p \leq 0.01$). At macroscopic examination, dark liver was found in 5/14 females and enlarged liver in 3/14 females compared to no incidences 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 fold increase in 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 microsomal proteins revealed a moderate increase in total P-450 content and in mean PROD, BROD and UDPGT activities (all statistically different from the control group $p \leq 0.01$). 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 liver hypertrophy and cell proliferation in the liver.

# I. MATERIALS AND METHODS

## A. MATERIALS:

**1. Test Material:** Phenobarbital
**Description:** White crystalline powder
**Lot/Batch:** 06100228
**Purity:** 99.6 %
**CAS:** 50-06-6
**Stability of test compound:** Stable for a period covering the study duration

**2. Vehicle and /or positive control:** Methylcellulose 400

**3. Test animals:**
**Species**: Rat
**Strain:** Wistar Rj: WI (IOPS HAN) – Female only
**Age:** 11 weeks approximately at start of treatment
**Weight at dosing:** 226 to 263 g
**Acclimation period:** 12 days
**Diet:** Certified rodent powdered and irradiated diet A04C-10 P1 from [redacted] ([redacted], [redacted], France), *ad libitum*
**Water:** Tap water, *ad libitum*
**Housing:** Animals were caged individually in suspended stainless steel wire mesh cages.
**Environmental conditions –**
**Temperature:** 20 – 24°C
**Humidity:** 40 – 70 %
**Air changes:** Approximately 10-15 changes per hour
**Photoperiod:** Alternating 12-hour light and dark cycles (7 am- 7 pm)

## B. STUDY DESIGN:

**1. In life dates:** Dosing period from November 19 to 26, 2007

**2. Animal assignment and treatment**

Phenobarbital was administered once daily by oral gavage for 7 days to a group of 15 females at a dose of 80 mg/kg/day in 0.5% aqueous solution of methycellulose 400. The dose level was selected on the basis of previous studies conducted with phenobarbital. A negative control group with the same number of animals received the vehicle only (0.5% aqueous solution of methycellulose 400). Animal housing and husbandry were in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals (Public Health Service, National Institute of Health, NIH publication N°86-23, revised 1985) and "Le Guide du Journal Officiel des Communautés Européennes L358, 18 Décembre 1986, N°86/609/CEE du 24 Novembre 1986".
A solution of BrdU at 80 mg of BrdU/100 mL of drinking water was administered to all animals for 7 days to allow liver cell proliferation evaluation.

**Table 5.5.4-5: Study design**

| Test group | Treatment | Dose level (mg/kg bw/day) | Animals assigned |
|---|---|---|---|
| 1 | Control | 0 | 15 |
| 2 | Phenobarbital | 80 | 15 |

Both Phenobarbital and BrdU concentrations were checked and found to be acceptable for use on the study, being in the range of 97 to 98 % of nominal concentration. Homogeneity of phenobarbital solution was also checked and found to be acceptable for use on the study.

**3. Diet and water**

Certified rodent powdered and irradiated diet A04CP1-10 from [redacted]. ([redacted], France) and filtered and softened tap water from the municipal water supply in individual bottles were available *ad libitum*, except prior to sacrifice when animals were diet fasted overnight. Routine analyses of food and water indicated that there was no contamination which could have compromised the study

**4. Statistics**

- Body weight change parameters,
- Terminal body weight, absolute and relative organ weight parameters,

Mean and standard deviation were calculated for each group and per time period for body weight change parameters.
The F test was performed to compare the homogeneity of group variances.
If the F test was not significant ($p>0.05$), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided).
If the F test was significant ($p\leq0.05$), mean of the exposed group was compared to the mean of the control group using the modified t-test (2-sided).

- Body weight and average food consumption/day parameters
- Total cytochrome P450 content

Mean and standard deviation were calculated for each group and per time period for average food consumption/day parameters.
The F test was performed to compare the homogeneity of group variances.
If the F test was not significant ($p>0.05$), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided).

If the F test was significant ($p\leq0.05$), data were transformed using the log transformation. If the F test on log transformed data was not significant ($p>0.05$), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided) on log transformed data.

If the F test was significant ($p\leq0.05$) even after log transformation, mean of the exposed group was compared to the mean of the control group using the modified t-test (2-sided).

If one or more group variance(s) equal 0, means were compared using non-parametric procedures.

- - Enzymatic activities and cell proliferation parameter

Mean of the exposed group will be compared to the mean of the control group using the exact Mann-Whitney test (2-sided).

Group means will be compared at the 5% and 1% levels of significance.
Statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics) except for liver enzyme parameters and cell proliferation parameters which were analyzed using SAS programs version 8.2.

## A. METHODS:

### 1. Observations

The animals were observed twice daily for moribundity and mortality (once daily on weekends or public holidays). Observed clinical signs were recorded at least once daily for all animals and detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility, and duration of any clinical signs were recorded.

### 2. Body weight

Each animal was weighed on study Day 1 and 7. Additionally, diet fasted animals were weighed before scheduled necropsy.

### 3. Food consumption

The weight of food supplied and of that remaining at the end of the food consumption period was recorded weekly for all animals during the treatment period. Any food spillage was noted.

### 4. Water consumption

Drinking water bottles containing BrdU were weighed on the first day of BrdU administration. Empty water bottles were weighed on the day of scheduled sacrifice (study Day 8).

### 5. Sacrifice and pathology

**Necropsy procedure – Organ sampling**
On study Day 8, all animals from all groups were sacrificed by exsanguination under deep anesthesia by inhalation of Isoflurane ([redacted], [redacted], France). Animals were diet fasted overnight prior to sacrifice.
All animals were necropsied. The necropsy included the examination of the external surfaces, all orifices and all major organs, tissues and body cavities. Brain and liver were weighed fresh at scheduled sacrifice only.
Duodenum and two central sections of the liver taken in the left and medial lobes were fixed by immersion in neutral buffered 10% formalin. The remaining portions of the liver from ten females from each group were kept for microsomal preparations and determination of total cytochrome P-450 content and isoenzyme activities.

**Histotechnology - Histopathology – Cell proliferation assessment**
Duodenum and the two central sections of the liver were embedded in paraffin wax.
Histological sections, stained with hematoxylin and eosin, were prepared and examined from all the animals.
For cell proliferation assessment, an immunohistochemical staining demonstrating the incorporation of BrdU and the determination of the labeling index were performed to assess hepatocytic cell cycling on all study animals. The immunohistochemical reaction included incubation with a monoclonal antibody raised against BrdU, amplification with a secondary biotinylated antibody and a streptavidin-horseradish peroxidase complex, detection of the complex with the chromogen diamino-benzidine (DAB) and nuclear counterstaining with hematoxylin.

The zonal labeling index, expressed as the number of BrdU-positive hepatocytes per thousand, was measured separately on random fields comprising at least 1000 centrilobular and 1000 periportal cells using an automated image analysis system. The mean labeling indexes (periportal, centrilobular and combined) and standard deviation were calculated for each zone and each group. The immunohistochemical staining for BrdU and determination of the labeling index were performed on all animals showing sufficient BrdU incorporation (estimated by water consumption or duodenum BrdU labeling).

**6. Hepatotoxicity testing:**

At final necropsy, the remaining portions of the liver from all surviving animals were homogenized for microsomal preparations in order to determine total cytochrome P-450 content and specific cytochrome P-450 isoenzyme profile (including EROD, BROD and PROD activities) to check the hepatotoxic potential of the test substance. Phase II enzymatic activities were also determined by measuring UDP-glucuronosyltransferase (UDPGT) with 4-nitrophenol as substrate. Results were compared to those generated with well known reference compounds.

## II. RESULTS AND DISCUSSION

### A. OBSERVATIONS:

Mortality:

One animal was found dead on day 5 of the study. The animal was discarded without necropsy. No cause of death could be established.

Clinical signs:

All animal receiving phenobarbital had reduced motor activity.

### B. BODY WEIGHT AND BODY WEIGHT GAIN:

Overall there was no mean body weight gain in the treated group compared to 7 g in the control group.

### C. FOOD and WATER CONSUMPTIONS:

Food and water consumption were not affected by the treatment with phenobarbital.

### D. :SACRIFICE AND PATHOLOGY

Higher liver weights (+20% approximately) were observed in animals treated with phenobarbital compared to controls. This was associated with enlarged (3/14) and dark (5/14) liver observed at the macroscopic examination and with hepatocellular hypertrophy in all animals at the microscopic examination.

**Table 5.5.4-6 Liver changes after 7 days of treatment with phenobarbital at 80 mg/kg bw/d**

| Dosage level (mg/kg bw/d) | 0 | 80 |
|---|---|---|
| **Liver weight** | | |
| Absolute (g) | 5.55±0.40 | 6.63±0.98** (+19%) |
| Body weight-relative | 2.47±0.16 | 3.02±0.36** (+22%) |
| Brain-relative | 296.35±20.81 | 358.66±49.18** (+21%) |
| **Microscopic pathology** | | |
| Diffuse centrilobular to panlobular hepatocellular hypertrophy | 0/15 | 14/14 |
| Diffuse mainly periportal hepatocellular vacuolation | 7/15 | 3/14 |

**: p≤0.01

**Cell proliferation**

Cell proliferation was assessed separately in the centrilobular and the periportal zones of the hepatic lobules. In the centrilobular and periportal areas, the mean BrdU labeling indexes were found to be 2 fold higher in treated animals, when compared to controls (p≤0.01). Centrilobular index was higher than periportal index in treated animals.

**Table 5.5.4-7 Mean BrdU labeling index in the liver after 7 days of treatment with phenobarbital at 80 mg/kg bw/d**

| Dosage level (mg/kg bw/d) | 0 | 80 |
|---|---|---|
| Number of animals | 15 | 14 |
| BrdU positive cells in the centrilobular zone | 21.73 ± 16.34 | 55.21 ± 43.31** |
| BrdU positive cells in the periportal zone | 16.70 ± 10.02 | 33.19 ± 18.22** |
| **Overall BrdU positive cells** | **19.22 ± 12.52** | **44.20 ± 27.39**** |

**: p≤0.01

**Hepatotoxicity testing**

Changes that were observed are described in Table 5.5.4-8. Assessment of total cytochrome P-450 content and microsomal proteins 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).

**Table 5.5.4-8 Results of the hepatotoxicity testing after 7 day of treatment with phenobarbital at 80 mg/kg bw/d**

| Dosage level (mg/kg bw/d) | 0 | 80 |
|---|---|---|
| N = | 10 | 10 |
| Total P-450 (nmol/mg prot.) | 0.95 ± 0.20 | 1.49 ± 0.38 ** (+ 57 %) |
| EROD (pmol/min/mg prot) | 38.25 ± 6.42 | 47.56 ± 9.75 * (+ 24 %) |
| PROD (pmol/min prot.) | 4.89 ± 0.61 | 26.36 ± 17.55 ** (+ 439 %) |
| BROD (pmol/min/mg prot.) | 4.91 ± 0.70 | 94.43 ± 62.94 ** (+ 1823 %) |
| UDPGT (nmol/min /mg Prot) | 6.99 ± 0.52 | 13.47 ± 1.66 ** (+ 93 %) |

* : p≤0.05; **: p≤0.01
(%) as compared to control

## III. CONCLUSION

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 liver hypertrophy and cell proliferation in the liver.

| | |
|---|---|
| **Report:** | **KIIA 5.5.4 /23; [redacted] 2011; M-408029-01** |
| Title: | Fluopyram (AE C 656948): Mechanistic investigation in the female rat by dietary administration for up to 7 days |
| Report No & Document No | SA 10240<br>M-408029-01 |
| **Guidelines:** | **No specific guideline** |
| **Deviations:** | **None** |
| **GLP** | **Yes** |

**Executive summary**

Fluopyram, (batch number: Mix-batch: 08528/0002: a light beige solid, 94.7% w/w), 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 at the start and end of the treatment period for each subgroup. 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).

# I. MATERIALS AND METHODS

## A. MATERIALS:

**1. Test Material:** AE C656948
**Description:** Light beige solid
**Lot/Batch:** Mix-Batch: 08528/0002
**Purity:** 94.7 %
**CAS:** 658066-35-4
**Stability of test compound:** Stable in rodent diet for a period covering the study duration

**2. Vehicle and /or positive control:** Phenobarbital (positive control)
**Description:** White crystalline powder
**Lot/Batch:** Lot No. 06100228
**Purity:** 99.6 %
**CAS:** 50-06-6
**Stability of test compound:** Stable in 0.5% aqueous solution of methylcellulose for a period covering the study duration

**3. Test animals:**
**Species**: Rat
**Strain:** Wistar Rj: WI (IOPS HAN) – Female only
**Age:** 10 weeks approximately at start of treatment
**Weight at dosing:** 218 to 263 g
**Acclimation period:** 12 days
**Diet:** Certified rodent powdered and irradiated diet A04C-10 P1 from [redacted] ([redacted], [redacted], France), *ad libitum,* except ffor overnight fasting prior to scheduled necropsy
**Water:** Tap water, *ad libitum*
**Housing:** Animals were caged individually in suspended stainless steel wire mesh cages.
**Environmental conditions –**
**Temperature:** 20 – 24°C
**Humidity:** 40 – 70 %
**Air changes:** Approximately 10-15 changes per hour
**Photoperiod:** Alternating 12-hour light and dark cycles (7 am- 7 pm)

## B. STUDY DESIGN:

**1. In life dates:** Dosing period from July 12 to 22, 2010

**2. Animal assignment and treatment**

Animals were assigned to dose groups using a randomization procedure by weight.

Seven groups of female rats were dosed for up to 7 days with the appropriate compound by the appropriate route of administration. Each group consisted of 30 female rats, 15 of which were sacrificed the morning after three days of treatment (Subgroup 1). The remaining 15 animals were sacrificed the morning after seven days of treatment (Subgroup 2).

Six groups were dosed by dietary administration. One group consisted of control animals that received untreated diet and the remaining five groups received fluopyram at the appropriate dietary level (30, 75, 150, 600 and 1500 ppm) at a constant level. The dose levels for fluopyram were set after evaluation of the results from previous studies conducted with fluopyram and were set after evaluation of the results from previous studies conducted with fluopyram and following discussions with the US (EPA) and Canadian (PMRA) authorities. The top dose level of 1500 ppm used in the present study represents the top dose level used in the rat cancer bioassay in which an increase in liver tumors was observed in the females.

The seventh group was dosed by oral gavage with 80 mg/kg/day phenobarbital suspended in 0.5% aqueous methylcellulose 400 using a dosing volume of 5ml/kg bodyweight. The volume administered to each rat was based on the most recent recorded body weight. A dose level of 80 mg/kg/day was chosen for phenobarbital as this dose is known to clearly induce liver cytochrome P-450 activity and cell proliferation in the rat.

Animal housing and husbandry were in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals (Public Health Service, National Institute of Health, NIH publication N°86-23, revised 1985) and "Le Guide du Journal Officiel des Communautés Européennes L358, 18 Décembre 1986, N°86/609/CEE du 24 Novembre 1986".

**Table 5.5.4-9: Study design**

| Test group | Concentration in diet (ppm) of AE C656948 | Dose per animal (study averages) | | Animals assigned | |
|---|---|---|---|---|---|
| | | Females Subgp 1 Days 1-3 (mg/kg bw/day) | Females Subgp 2 Days 1-7 (mg/kg bw/day) | Females Subgp 1 | Females Subgp 2 |
| 1 | 0 | 0 | 0 | 15 | 15 |
| 2 | 30 | 2.4 | 2.3 | 15 | 15 |
| 3 | 75 | 6.2 | 5.6 | 15 | 15 |
| 4 | 150 | 12.0 | 11.6 | 15 | 15 |
| 5 | 600 | 46.1 | 44.1 | 15 | 15 |
| 6 | 750 | 117.6 | 118.5 | 15 | 15 |
| 7 | Phenobarbital 80 mg/kg bw/day by gavage | - | - | 15 | 15 |

Subgp = Subgroup

**3. Diet preparation and analysis**

Fluopyram (AE C656948) was incorporated into the diet by dry mixing to provide the required concentrations. There was one preparation for each concentration used in the study. The stability was demonstrated in an earlier study at concentrations of 20 and 10000 ppm for a time which covered the period of usage and storage for the current study. Homogeneity of test substance in diet was verified on the lowest and highest concentrations to demonstrate adequate formulation procedures. The mean value obtained in homogeneity check was used as measured concentration. For the remaining dietary levels, concentration was checked.

A single formulation of phenobarbital was prepared by suspending the test substance (w/w) in a 0.5% aqueous solution of methylcellulose. The stability of phenobarbital at 8 g/l was demonstrated in an earlier study, which covered the period of usage and storage for the current study. Homogeneity of phenobarbital in aqueous methylcellulose was verified to demonstrate adequate formulation formulation procedures. The mean value obtained in homogeneity check was used as measured concentration.

**4. Statistics**

4.1 Variables analyzed

- Body weight parameters
- Body weight change parameters calculated according to time intervals
- Average food consumption/day parameters calculated according to time intervals
- Terminal body weight, absolute and relative organ weights parameters
- Total cytochrome P450 content and liver enzyme activities
- Cell proliferation parameters
- Gene transcript analysis

4.2 Statistical methods

Group means were compared at the 5% and 1% levels of significance.

With the exception of those used for the cell proliferation data, all statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics). SAS programs (version 9) were used for the cell proliferation data.

Fluopyram Treatment

- Body weight change parameters,
- Terminal body weight, absolute and relative organ weight parameters,

Mean and standard deviation were calculated for each group and per time period for body weight change parameters.

The Bartlett test was performed to compare the homogeneity of group variances.
If the Bartlett test was not significant ($p>0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p>0.05$), the group means were considered to be homogeneous and no further analysis will be performed. If the ANOVA was significant ($p\leq0.05$), means of the exposed groups were compared to the mean of the control group using the Dunnett test (2-sided).
If the Bartlett test was significant ($p\leq0.05$), group means were compared using the non-parametric Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p>0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p\leq0.05$), means of the exposed groups were compared to the mean of the control group using the Dunn test (2-sided).

- Body weight and average food consumption/day parameters
- Total cytochrome P450 content and liver enzyme activities
- Gene transcript analyses

Mean and standard deviation were calculated for each group and per time period for average food consumption/day parameters.

The Bartlett test was performed to compare the homogeneity of group variances.
If the Bartlett test was not significant ($p>0.05$), means were compared using the analysis of variance (ANOVA). If the ANOVA was not significant ($p>0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant ($p\leq0.05$), means of the exposed groups were compared to the mean of the control group using the Dunnett test (2-sided).
If the Bartlett test was significant ($p\leq0.05$), data were transformed using the log transformation.
- If the Bartlett test on log transformed data was not significant ($p>0.05$), means were compared using the ANOVA on log transformed data. If the ANOVA on log transformed data was not significant ($p>0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA on log transformed data was significant ($p\leq0.05$), means of the exposed groups were compared to the mean of the control group using the Dunnett test (2-sided) on log transformed data.
- If the Bartlett test was significant ($p\leq0.05$) even after log transformation, group means were compared using the non-parametric Kruskal-Wallis test. If the Kruskal-Wallis test was not significant ($p>0.05$), the group means were considered to be homogeneous and no further analysis was performed. If the Kruskal-Wallis test was significant ($p\leq0.05$), means of the exposed groups were compared to the mean of the control group using the Dunn test (2-sided).

- Cell Proliferation analyses

  The Levene test was performed to compare the homogeneity of group variances.
  If the Levene test was not significant ($p>0.05$), means of the exposed groups were compared to the mean of the control group using the Dunnett test (1-sided).
  If the Levene test was significant ($p\leq0.05$), data were transformed using the log transformation.
  If the Levene test on log transformed data was not significant ($p>0.05$), means of the exposed groups were compared to the mean of the control group using the Dunnett test (1-sided) on log transformed data.
  If the Levene test was significant ($p\leq0.05$) even after log transformation, means of the exposed groups were compared to the mean of the control group using the Dunn test (1-sided).
  If one or more group variance(s) equal 0, means were compared using non-parametric procedures.

<u>Phenobarbital Treatment</u>

- Body weight change parameters,
- Terminal body weight, absolute and relative organ weight parameters,

Mean and standard deviation were calculated for each group and per time period for body weight change parameters.
The F test was performed to compare the homogeneity of group variances.
If the F test was not significant (p>0.05), the mean of the exposed group was compared to the mean of the control group using the t-test (2-sided).
If the F test was significant (p≤0.05), the mean of the exposed group was compared to the mean of the control group using the modified t-test (2-sided).

- Body weight and average food consumption/day parameters
- Total cytochrome P450 content and liver enzyme activities
- Gene transcript analyses

  Mean and standard deviation were calculated for each group and per time period for average food consumption/day parameters.
  The F test was performed to compare the homogeneity of group variances.
  If the F test was not significant (p>0.05), the mean of the exposed group was compared to the mean of the control group using the t-test (2-sided).
  If the F test was significant (p≤0.05), the data were transformed using log transformation. If the F test on log transformed data was not significant (p>0.05), the mean of the exposed group was compared to the mean of the control group using the t-test (2-sided) on log transformed data.
  If the F test was significant (p≤0.05) even after log transformation, the mean of the exposed group was compared to the mean of the control group using the modified t-test (2-sided).

- Cell proliferation analyses

  The F test was performed to compare the homogeneity of group variances.
  If the F test was not significant (p>0.05), mean of the phenobarbital exposed group was compared to the mean of the control group using the t-test (1-sided).
  If the F test was significant (p≤0.05), data were transformed using the log transformation. If the F test on log transformed data was not significant (p>0.05), mean of the Phenobarbital exposed group was compared to the mean of the control group using the t-test (1-sided) on log transformed data.
  If the F test was significant (p≤0.05), even after log transformation, mean of the exposed group was compared to the mean of the control group using the exact Mann-Whitney test (1-sided).

If one or more group variance(s) equaled 0, means were compared using non-parametric procedures.

## C. METHODS:

### 1. Observations

The animals were observed twice daily for moribundity and mortality (once daily on weekends or public holidays). Observed clinical signs were recorded at least once daily for all animals and detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility, and duration of any clinical signs were recorded.

**2. Body weight**

Each animal was weighed during the acclimatization period. Body weights were also measured on study Day 1 and 3 for Subgroup 1 and on study Day 1 and 7 for Subgroup 2. Additionally, diet fasted animals were weighed before scheduled necropsy (terminal body weight).

**3. Food consumption and compound intake**

The weight of food supplied and of that remaining at the end of the food consumption period was recorded on study Day 3 for all animals in Subgroup 1 and on study Day 7 for all animals in Subgroup 2. Any food spillage was noted. From these records, the mean achieved dosage intake in mg/kg/day for each Subgroup was calculated.

**4. Sacrifice and pathology**

**4.1 Necropsy procedure – Organ sampling**

On study Day 4 (Subgroup 1) and study Day 8 (Subgroup 2), all animals were sacrificed by exsanguination under deep anesthesia by inhalation of Isoflurane ([redacted], France). Animals were diet fasted overnight prior to sacrifice.
All animals were necropsied. The necropsy included the examination of the external surfaces, all orifices and all major organs, tissues and body cavities. Brain and liver were weighed fresh at scheduled sacrifice only.
Duodenum and two central sections of the liver taken from the left and medial lobes from each animal were fixed by immersion in neutral buffered 10% formalin. In addition, a piece of the median and the left lobe of the liver from each animal were collected and flash frozen in liquid nitrogen, were stored at approximately -74°C + 10°C until used for qPCR investigations. From the Subgroup 2 animals, the remaining portions of liver from each of 5 randomly selected females per group were weighed and homogenized for microsomal preparations.

**4.2 Histotechnology**

**4.2.1 Conventional Histopathological examination**

Duodenum and the two central sections of the liver were embedded in paraffin wax.
Histological sections, stained with hematoxylin and eosin, were prepared for each animal in all groups.

**4.2.2 Ki67 staining for Cell proliferation assessment**

For each animal in each group a section of a formalin-fixed paraffin-embedded block containing 2 liver samples and one sample of duodenum was prepared. The duodenum was included to serve as a positive control for staining as it has a high rate of cell proliferation. The immunohistochemical reaction included incubation with a monoclonal antibody raised against Ki67, amplification with a secondary biotinylated antibody and a streptavidin-horseradish complex, detection of the complex with diamino-benzidine (DAB) and nuclear counter staining with hematoxylin.

**4.3 Histopathology**

Histopathological examinations were performed on the liver samples from all animals in all groups.

**4.4 Cell proliferation assessment**

The immunohistochemical staining for Ki67 and determination of the labeling index was performed on all surviving animals showing sufficient Ki67 staining (estimated by duodenal Ki67

labeling) to assess cell cycling in the liver. The zonal labeling index, expressed as the number of Ki67-positive hepatocytes per thousand cells, were measured separately on random fields comprising of at least 1000 centrilobular and perilobular cells using an automatic image analysis system. The mean and standard deviation were calculated for each group.

### 4.5 Hepatotoxicity testing

At final necropsy (ie Day 8), the remaining portions of the liver from were five randomly chosen females per treatment group (Subgroup 2) were weighed and homogenized for microsomal preparations in order to determine total cytochrome P-450 content and specific cytochrome P-450 isoenzyme profile (including EROD, BROD and PROD activities) and UDPGT specific isoenzyme profiles.

#### 4.5.1 Total cytochrome P-450 content

Total cytochrome P-450 content in microsomal preparations was determined by spectrophotometry (Cary Win UV version 3.0 (182)) using a reduced CO differential spectrum. A single quantification was performed for each sample.

#### 4.5.2 Enzymatic activities

Cytochrome P-450s and typical inducing agents:

| Family | Enzymatic activity | Activity | Typical inducing agents |
|---|---|---|---|
| CYP 1A1<br>1A2 | } EROD | activation of mutagens and carcinogens | β-naphtoflavone |
| CYP 2<br>2B1<br>2B2 | } PROD | detoxication of drugs and chemicals | phenobarbital |
| 2E | | activation of nitrosamines | isoniazid |
| CYP 3A1 & 3A2 | BROD | detoxication of drugs and chemicals | pregnenolone<br>16 α - carbonitrile<br>phenobarbital |

Specific cytochrome P-450 enzymatic activities were evaluated by spectrofluorimetry (SAFAS SP2000 version 6.10.7.4) using the following substrates:

- ethoxyresorufin (EROD)
- pentoxyresorufin (PROD)
- benzoxyresorufin (BROD)

Ethoxyresorufin is a highly selective substrate for the isoform 1A, the isoform 2B metabolizes preferentially the O-dealkylation of pentoxyresorufin, while the benzoxyresorufin O-debenzylation is mainly metabolized by the isoform 3A. Cytochrome P-450 dependent dealkylation of resorufin derivatives was followed over a period of 2, 5 or 7 minutes at 37°C.

Phase II enzymatic activities were also determined by measuring UDP-glucuronosyltransferase (UDPGT) with 4-nitrophenol as substrate (method adapted from Zakim and Vessey. The enzymatic kinetic (disappearance of the colored 4-nitrophenol) was followed at 405 nm during 3 min. at 30°C. Three replicates from each sample were assayed. UDPGT with bilirubin as substrate was also

determined using a spectrophotometry method (adapted from Heirwegh et al.) consisting in the determination of conjugated bile pigments after its conversion into azo-pigment derivatives. Absorbance was measured at 530 nm. Three replicates from each sample were assayed.

## 4.6 Quantitative PCR Analyses

### 4.6.1 Total RNA purification

Total cytoplasmic RNA was isolated from the liver of all surviving individual control and treated animals using RNeasy Midi kits (Qiagen). RNA quality controls were performed based on the ribosomal RNA electrophoretic profiles using a Bioanalyser (Agilent Technologies).

### 4.6.2 Quantitative PCR

Ten µg of total RNA was used for Reverse transcription (RT) using a High Capacity cDNA Archive kit (Applied Biosystems). The assay was performed in duplicate using Taqman assays (Assay on demand, Applied Biosystems), 1/50 diluted first strand cDNA, Fast Start Universal Probe Master mix (Roche) on an ABI prism 7900 HT machine (Applied Biosystems). For each gene transcript measured, a negative control condition was included in which H2O MQ was used as template instead of first strand cDNA.

The list of Taqman assays used is as follows:

| Gene (Major function) | Abbreviation | Refset ID | Taqman assay ID (Applied Biosystems) |
|---|---|---|---|
| METABOLISM: Phase I | | | |
| P450 (cytochrome)oxidoreductase | POR | NM_031576.1 | Rn00580820_m1 |
| Cytochrome P450 1a1 | 1a1 | NM_012540.2 | Rn00487218_m1 |
| Cytochrome P450 2b1 | Cyp2b2 | 275205* | Cyp2b1_tc5 |
| Cytochrome P450 3a3 | Cyp3a3 (Cyp3a23) | NM_013105.2 | Rn01640761_g1 |
| Cytochrome P450 4a1 | Cyp4a1 | NM_175837.1 | Rn00598510_m1 |
| METABOLISM: Phase II | | | |
| Glutathione S-transferase A2 | Gsta2 | NM_017013.4 | Rn00566636_m1 |
| Glutathione S-transferase mu3 | Gstm4 | NM_020540.1 | Rn01789233_m1 |
| UDP glucoronosyltransferase 2 family, polypeptide B1 | Udpgtr2 | NM_173295.1<br>NM_057105.3 | Rn00756519_m1 |
| Epoxide hydrolase 1, microsomal | Ephx1 | NM_012844.2 | Rn00563349_m1 |
| Epoxide hydrolase 2, cytoplasmic | Ephx2 | NM_022936.1 | Rn00576023_m1 |
| Sulfotranferase family 1E, member 1 | Sult1e1 | NM_012883.1 | Rn00820646_g1 |
| CELL PROLIFERATION/APOPTOSIS | | | |
| Epithelial cell adhesion molecule | Tacstd1 | NM_138541.1 | Rn01473202_m1 |
| Growth arrest and DNA-damage-inducible 45 beta | Gadd45b | NM_001008321.1 | Rn01452530_g1 |
| Retinoblastoma 1 | Rb1 | XM_344434.3 | Rn01753308_m1 |

***:** Transcript made to order

Beta-microglobulin (B2m; Refset ID: NM_012512.1; Taqman assay ID: Rn00560865_m1) was selected as reference gene for the quantitative calculations of transcripts in the liver. The relative quantity (RQ) value of each test transcript was calculated using the following formula:

$\Delta\Delta Ct = (Ct_{test} - Ct_{B2m})_{treated} - (Ct_{test} - Ct_{B2m})_{control}$
$RQ = 2^{-\Delta\Delta Ct}$
where Ct is the threshold cycle at which PCR amplification started to be significantly different from the background signal. As a Ct of ≥35 indicates that a gene is poorly expressed in the tissue investigated, any subsequent RQ data generated from such a Ct are considered as non-relevant due to an increased risk of contamination.

## II. RESULTS AND DISCUSSION

### A. OBSERVATIONS:

Mortality:

There was no mortality in any group during the course of the study.

Clinical signs:

**Three day treatment (Subgroup 1):**

Fluopyram: There were no treatment-related clinical signs during the three day treatment period.

Phenobarbital: All females displayed reduced motor activity starting from Day 2 of treatment. In addition, three females had ocular discharge in either one eye or both eyes on Day 3 of treatment.

**Seven day treatment (Subgroup 2):**

Fluopyram: There were no treatment-related clinical signs during the seven day treatment period.

Phenobarbital: All females displayed reduced motor activity starting from Day 1 of treatment. Two females had ocular discharge starting from Day 2 of treatment.

### B. BODY WEIGHT AND BODY WEIGHT GAIN:

**Three day treatment (Subgroup 1):**

Fluopyram: There were no treatment-related changes to any of the body weight parameters between Day 1 and Day 3.

Phenobarbital: There were no relevant changes in any of the body weight parameters between Day 1 and Day 3 compared to the controls.

**Seven day treatment (Subgroup 2):**

Fluopyram: There were no relevant changes in any of the body weight parameters between Day 1 and Day 7 compared to the controls.

Phenobarbital: Between Days 1 and 7, there was a reduced mean absolute body weight gain compared to the control group (10g compared to 13g in the control group; -23%, not statistically significant).

## C. FOOD CONSUMPTION AND COMPOUND INTAKE:

There were no treatment-related effects on food consumption for either Fluopyram or Phenobarbital following treatment for either 3 or 7 days

The mean achieved dietary intakes of Fluopyram expressed in mg/kg/day received by the females during the study were as follows:

**Table 5.5.4-10: Mean achieved intake of fluopyram**

| | Mean achieved dietary intake of fluopyram | |
|---|---|---|
| **Diet Concentration (ppm)** | **SUBGROUP 1**<br>**Days 1 – 3 (mg/kg/day)** | **SUBGROUP 2**<br>**Days 1 – 7 (mg/kg/day)** |
| **30** | **2.4** | **2.3** |
| **75** | **6.2** | **5.6** |
| **150** | **12.0** | **11.6** |
| **600** | **46.1** | **44.1** |
| **1500** | **117.6** | **118.5** |

## D. SACRIFICE AND PATHOLOGY

### 1. Terminal body weight and organ weight

**Three day treatment (Subgroup 1):**

Fluopyram: There was no change in mean terminal body weight in treated animals when compared to the controls. The mean absolute and mean liver to body weight ratio were statistically significantly higher from 600 ppm when compared to controls. In addition, the mean liver to brain weight ratio was also statistically significantly higher at 1500 ppm when compared to controls. These liver weights changes were considered to be toxicologically relevant.

Phenobarbital: There was no change in mean terminal body weight in treated animals when compared to the controls. Mean absolute and relative liver weights were statistically significantly higher when compared to controls. These liver weights changes were considered to be toxicologically relevant.

**Table 5.5.4-11: Mean absolute and relative liver weight changes following 3 day treatment with fluopyram or phenobarbital**

| Mean liver weight ± SD at scheduled 3 Day sacrifice (% change when compared to controls) | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Sex** | **Females** | | | | | | |
| **Dose level of Fluopyram (ppm)** | **0** | **30** | **75** | **150** | **600** | **1500** | **PB (80 mg/kg)** |
| **Mean absolute liver weight (g)** | **5.84 ±0.30** | **5.93 ±0.35 (+2%)** | **5.97 ±0.40 (+2%)** | **6.16 ±0.26 (+5%)** | **6.23* ±0.49 (+7%)** | **6.84** ±0.38 (+17%)** | **6.36** ±0.57 (+9%)** |
| **Mean liver to body weight ratio (%)** | **2.69 ±0.08** | **2.70 ±0.15 (0%)** | **2.75 ±0.13 (+2%)** | **2.79 ±0.10 (+4%)** | **2.83* ±0.17 (+5%)** | **3.15** ±0.17 (+17%)** | **2.93** ±0.22 (+9%)** |
| **Mean liver to brain weight ratio (%)** | **313.30 ±17.26** | **318.52 ±18.34 (+2%)** | **321.00 ±25.37 (+2%)** | **331.36 ±18.03 (+6%)** | **333.10 ±25.17 (+6%)** | **365.96** ±32.54 (+17%)** | **341.02** ±28.83 (+9%)** |

*** p≤0.05; ** p≤0.01**

**Seven day treatment (Subgroup 2):**

Fluopyram: There was no change in mean terminal body weight in treated animals when compared to the controls. Mean absolute and relative liver weights were statistically significantly higher at 1500 ppm when compared to controls. These liver weight changes were considered to be toxicologically relevant.

Phenobarbital: There was no change in mean terminal body weight in treated animals when compared to the controls. Mean liver to body weight ratio was statistically significantly higher when compared to controls. This change was considered to be toxicologically relevant.

**Table 5.5.4-12: Mean absolute and relative liver weight changes following 7 day treatment with fluopyram or phenobarbital**

| Mean liver weight ± SD at scheduled 7 Day sacrifice (% change when compared to controls) | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Sex** | **Females** | | | | | | |
| **Dose level of Fluopyram (ppm)** | **0** | **30** | **75** | **150** | **600** | **1500** | **PB (80 mg/kg)** |
| **Mean absolute liver weight (g)** | **6.18 ±0.91** | **5.88 ±0.53 (-5%)** | **5.96 ±0.43 (-4%)** | **5.96 ±0.35 (-4%)** | **6.29 ±0.49 (+2%)** | **7.17** ±0.51 (+16%)** | **6.66 ±0.37 (+8%)** |
| **Mean liver to body weight ratio (%)** | **2.67 ±0.34** | **2.52 ±0.13 (-5%)** | **2.59 ±0.14 (-3%)** | **2.61 ±0.20 (-2%)** | **2.76 ±0.16 (+3%)** | **3.16** ±0.16 (+18%)** | **2.95** ±0.17 (+11%)** |
| **Mean liver to brain weight ratio (%)** | **332.98 ±53.94** | **317.53 ±27.19 (-5%)** | **321.09 ±23.25 (-4%)** | **320.04 ±23.75 (-4%)** | **336.49 ±22.32 (+1%)** | **392.26** ±29.24 (+18%)** | **361.88 ±20.36 (+9%)** |

**** p≤0.01**

2. Gross pathology

**Three day treatment (Subgroup 1):**

Enlarged liver was found in 2/15 females at 1500 ppm fluopyram. There were no macroscopic changes recorded for the phenobarbital treated females.

**Table 5.5.4-13: Macroscopic changes in the liver following 3 days treatment with fluopyram or phenobarbital**

| INCIDENCE AND SEVERITY OF MACROSCOPIC CHANGES IN THE LIVER- SCHEDULED 3 DAY SACRIFICE | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Sex** | **Females** | | | | | | |
| **Dose level of Fluopyram (ppm)** | **0** | **30** | **75** | **150** | **600** | **1500** | **PB (80 mg/kg)** |
| **Number examined** | **15** | **15** | **15** | **15** | **15** | **15** | **15** |
| **Enlarged** | **0** | **0** | **0** | **0** | **0** | **2** | **0** |

**Seven day treatment (Subgroup 2):**

Enlarged liver was found in 3/15 females at 1500 ppm fluopyram. There were no macroscopic changes recorded for the phenobarbital treated females.

**Table 5.5.4-14: Macroscopic changes in the liver following 7 days treatment with fluopyram or phenobarbital**

| INCIDENCE AND SEVERITY OF MACROSCOPIC CHANGES IN THE LIVER- SCHEDULED 7 DAY SACRIFICE | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Sex** | **Females** | | | | | | |
| **Dose level of Fluopyram (ppm)** | **0** | **30** | **75** | **150** | **600** | **1500** | **PB (80 mg/kg)** |
| **Number examined** | **15** | **15** | **15** | **15** | **15** | **15** | **15** |
| **Enlarged** | **0** | **0** | **0** | **0** | **0** | **3** | **0** |

3. Microscopic pathology

**Three day treatment (Subgroup 1):**

Fluopyram: Minimal centrilobular to panlobular hepatocellular hypertrophy was found for 6/15 females at 1500 ppm. In addition, an increased number of mitoses in hepatocytes was found at the same dose level for 4/15 females.

Phenobarbital: Minimal centrilobular to panlobular hepatocellular hypertrophy was found for 3/15 females. In addition, an increased number of mitoses in hepatocytes was found for 3/15 females.

**Table 5.5.4-15: Microscopic changes in the liver following 3 days treatment with fluopyram or phenobarbital**

| INCIDENCE AND SEVERITY OF MICROSCOPIC CHANGES IN THE LIVER- SCHEDULED 3 DAY SACRIFICE | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Sex** | **Females** | | | | | | |
| **Dose level of Fluopyram (ppm)** | **0** | **30** | **75** | **150** | **600** | **1500** | **PB (80 mg/kg)** |
| **Number examined** | **15** | **15** | **15** | **15** | **15** | **15** | **15** |
| Hepatocellular hypertrophy: centrilobular to panlobular | | | | | | | |
| **Minimal** | **0** | **0** | **0** | **0** | **0** | **6** | **3** |
| Total | 0 | 0 | 0 | 0 | 0 | 6 | 3 |
| Increased number of mitoses | | | | | | | |
| **Present** | **0** | **0** | **0** | **0** | **0** | **4** | **3** |
| Total | 0 | 0 | 0 | 0 | 0 | 4 | 3 |

**Seven day treatment (Subgroup 2):**

Fluopyram: Minimal to slight centrilobular to panlobular hepatocellular hypertrophy was found from 600 ppm (1/15 females at 600 ppm; 14/15 females at 1500 ppm).

Phenobarbital: Minimal to slight centrilobular to panlobular hepatocellular hypertrophy in 9/15 females was found as well as an increased number of mitoses in hepatocytes in 3/15 females.

**Table 5.5.4-16: Microscopic changes in the liver following 7 days treatment with fluopyram or phenobarbital**

| INCIDENCE AND SEVERITY OF MICROSCOPIC CHANGES IN THE LIVER- SCHEDULED 7 DAY SACRIFICE | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Sex** | **Females** | | | | | | |
| **Dose level of Fluopyram (ppm)** | **0** | **30** | **75** | **150** | **600** | **1500** | **PB (80 mg/kg)** |
| **Number examined** | **15** | **15** | **15** | **15** | **15** | **15** | **15** |
| Hepatocellular hypertrophy: centrilobular to panlobular | | | | | | | |
| **Minimal** | **0** | **0** | **0** | **0** | **1** | **6** | **6** |
| **Slight** | **0** | **0** | **0** | **0** | **0** | **8** | **3** |
| Total | 0 | 0 | 0 | 0 | 1 | 14 | 9 |
| Increased number of mitoses | | | | | | | |
| **Present** | **0** | **0** | **0** | **0** | **0** | **0** | **3** |
| Total | 0 | 0 | 0 | 0 | 0 | 0 | 3 |

4. Cell proliferation

**Three day treatment (Subgroup 1):**

Fluopyram: Dose-related increases in the centrilobular, perilobular and, therefore, the global proliferation indexes were observed, which were statistically significantly higher from 150 ppm when compared to controls.

**Table 5.5.4-17: Mean cell proliferation index following 3 days treatment with fluopyram**

| Mean cell proliferation index ± SD following three days treatment (% change when compared to controls) | | | | | |
|---|---|---|---|---|---|
| **Test Substance** | **Dose** | | **Centrilobular** | **Perilobular** | **Total** |
| **Control** | **0 ppm** | **N** | **15** | **15** | **15** |
| | | **Mean ± SD** | 14.6 ± 7.4 | 11.1 ± 5.1 | 12.8 ± 5.8 |
| **Fluopyram** | **30 ppm** | **N** | **14** | **14** | **14** |
| | | **Mean ± SD** | 11.8 ± 8.0 | 10.7 ± 5.8 | 11.2 ± 6.4 |
| | | **% Change** | **-19%** | **-3.5%** | **-12.5%** |
| | **75 ppm** | **N** | **15** | **15** | **15** |
| | | **Mean ± SD** | 13.4 ± 5.9 | 15.4 ± 8.2 | 14.4 ± 5.8 |
| | | **% Change** | **-8.2%** | **39.3%** | **12.3%** |
| | **150 ppm** | **N** | **15** | **15** | **15** |
| | | **Mean ± SD** | 25.1 ± 11.1* | 22.6 ± 13.4** | 23.8 ± 10.6** |
| | | **% Change** | **71.9%** | **104.2%** | **86.0%** |
| | **600 ppm** | **N** | **15** | **15** | **15** |
| | | **Mean ± SD** | 57.3 ± 20.1** | 36.6 ± 15.7** | 47.0 ± 15.8** |
| | | **% Change** | **293.8%** | **230.7%** | **266.6%** |
| | **1500 ppm** | **N** | **15** | **15** | **15** |
| | | **Mean ± SD** | 99.5 ± 62.3** | 67.4 ± 30.0** | 83.4 ± 38.3** |
| | | **% Change** | **583.2%** | **509.0%** | **551.2%** |

* p≤0.05; ** p≤0.01

In the above table the presented % changes do not always calculate exactly from the presented mean data. This is due to rounding-up differences.

Phenobarbital: Centrilobular, perilobular and, therefore, the global proliferation indexes were statistically significantly higher, when compared to controls. The magnitude of the response was greater in the centrilobular region (217.7% increase compared to the corresponding controls) than that observed in the perilobular region (58% increase compared to the corresponding controls).

**Table 5.5.4-18: Mean cell proliferation index following 3 days treatment with phenobarbital**

| Mean cell proliferation index ± SD following three days treatment (% change when compared to controls) | | | | | |
|---|---|---|---|---|---|
| **Test substance** | **Dose** | | **Centrilobular** | **Perilobular** | **Total** |
| **Control** | **0 ppm** | **N** | **15** | **15** | **15** |
| | | **Mean ± SD** | 14.6 ± 7.4 | 11.1 ± 5.1 | 12.8 ± 5.8 |
| **Phenobarbital** | **80 mg/kg/day** | **N** | **14** | **14** | **14** |
| | | **Mean ± SD** | 46.25 ± 36.24** | 17.48 ± 8.95* | 31.87 ± 19.80** |
| | | **% Change** | **217.7%** | **58.0%** | **148.8%** |

* p≤0.05; ** p≤0.0 In the above table the presented % changes do not always calculate exactly from the presented mean data. This is due to rounding-up differences.

**Seven day treatment (Subgroup 2):**

Fluopyram: Centrilobular, perilobular and, therefore, the global proliferation indexes were statistically significantly higher from 150 ppm when compared to controls.

**Table 5.5.4-19: Mean cell proliferation index following 7 days treatment with fluopyram**

| Mean cell proliferation index ± SD following seven days treatment (% change when compared to controls) | | | | | |
|---|---|---|---|---|---|
| **Test Substance** | **Dose** | | **Centrilobular** | **Perilobular** | **Total** |
| **Control** | **0 ppm** | **N** | **15** | **15** | **15** |
| | | **Mean ± SD** | 8.3 ± 5.3 | 10.5 ± 6.1 | 9.4 ± 5.1 |
| **Fluopyram** | **30 ppm** | **N** | **15** | **15** | **15** |
| | | **Mean ± SD** | 11.2 ± 7.6 | 15.3 ± 11.2 | 13.2 ± 8.6 |
| | | **% Change** | **34.5%** | **45.6%** | **40.7%** |
| | **75 ppm** | **N** | **15** | **15** | **15** |
| | | **Mean ± SD** | 12.0 ± 6.1 | 10.3 ± 5.1 | 11.1 ± 5.1 |
| | | **% Change** | **43.5%** | **NC** | **18.5%** |
| | **150 ppm** | **N** | **15** | **15** | **15** |
| | | **Mean ± SD** | 20.4 ± 13.0** | 18.4 ± 8.1** | 19.4 ± 9.6** |
| | | **% Change** | **144.5%** | **75.2%** | **106.0%** |
| | **600 ppm** | **N** | **15** | **15** | **15** |
| | | **Mean ± SD** | 27.8 ± 12.1** | 27.0 ± 13.0** | 27.4 ± 9.8** |
| | | **% Change** | **234.1%** | **157.6%** | **191.7%** |
| | **1500 ppm** | **N** | **15** | **15** | **15** |
| | | **Mean ± SD** | 32.2 ± 19.6** | 34.9 ± 17.2** | 33.5 ± 15.0** |
| | | **% Change** | **286.3%** | **232.5%** | **256.6%** |

NC: no change; ** p≤0.01
In the above table the presented % changes do not always calculate exactly from the presented mean data. This is due to rounding-up differences.

Phenobarbital: Centrilobular and the global proliferation indexes were statistically significantly higher when compared to controls. The magnitude of response was greater in the centrilobular region (603.4% increase compared to controls) compared to that recorded for the perilobular region (54.7% increase compared to controls).

**Table 5.5.4-20: Mean cell proliferation index following 7 days treatment with phenobarbital**

| Mean cell proliferation index ± SD following seven days treatment (% change when compared to controls) | | | | | |
|---|---|---|---|---|---|
| **Test Substance** | **Dose** | | **Centrilobular** | **Perilobular** | **Total** |
| **Control** | **0 ppm** | **N** | **15** | **15** | **15** |
| | | **Mean ± SD** | 8.3 ± 5.3 | 10.5 ± 6.1 | 9.4 ± 5.1 |
| **Phenobarbital** | **80 mg/kg/day** | **N** | **15** | **15** | **15** |
| | | **Mean ± SD** | 58.6 ± 33.9** | 16.2 ± 10.2 | 37.4 ± 19.6** |
| | | **% Change** | **603.4%** | **54.7%** | **297.9%** |

* p≤0.05; ** p≤0.01
In the above table the presented % changes do not always calculate exactly from the presented mean data. This is due to rounding-up differences.

**5. Hepatotoxicity testing**

Investigation of hepatotoxicity was only conducted on 5 randomly chosen females/group from those animals dosed for 7 days (Subgroup 2).

Fluopyram: No statistically significant changes in total P450 content or enzyme activity was observed up to 150 ppm. Statistically significant increases in PROD ($p \leq 0.05$ at 600 ppm and $p \leq 0.01$ at 1500 ppm), BROD and both UDPGT isoforms ($p \leq 0.01$) were observed from 600 ppm. At 1500 ppm, total P450 content was also statistically significantly increased ($p \leq 0.05$). In addition, EROD activity was statistically significantly increased ($p \leq 0.01$) at 1500 ppm, however the increase (63%) was considered marginal in comparison with the positive control, beta-naphthoflavone, which induced EROD by 800% compared to controls (9).

Phenobarbital: A statistically significant increase ($p \leq 0.05$) in total P450 was recorded as well as statistically significant increases BROD ($p \leq 0.05$), PROD ($p \leq 0.01$) and UDPGT-nitrophenol ($p \leq 0.01$) activity.

**Table 5.5.4-21: Mean cytochrome P-450 content and enzymatic activities in the liver following 7 days treatment with fluopyram or phenobarbital**

| Fluopyram dose | Mean Increase Compared to Controls (Statistical Significance) | | | | | |
|---|---|---|---|---|---|---|
| | Total P450 Content | EROD | BROD | PROD | UDPGT-4-nitrophenol | UDPGT-bilirubin |
| 30 ppm | x 0.93 | x 1.05 | x 1.09 | x 1.34 | x 1.05 | x 1.05 |
| 75 ppm | NC | x 0.94 | x 1.17 | x 1.46 | x 1.10 | x 1.28 |
| 150 ppm | x 1.11 | x 1.02 | x 1.44 | x 1.41 | x 1.27 | x 1.44 |
| 600 ppm | x 1.02 | x 1.12 | x 2.42 (**) | x 2.16 (*) | x 1.52 (**) | x 1.91 (**) |
| 1500 ppm | x 1.34 (*) | x 1.62 (**) | x 5.89 (**) | x 4.56 (**) | x 3.01 (**) | x 2.65 (**) |
| Phenobarbital 80 mg/kg/day | x 1.65 (*) | x 1.12 | x 27.7 (*) | x 10.59 (**) | x 1.86 (**) | x 1.37 |

**NC: no change; * $p \leq 0.05$; ** $p \leq 0.01$**

## 6. Gene Transcript Analyses

Three day treatment (Subgroup 1):

Fluopyram: At 30 ppm, no statistically significant changes in the expression of any of the genes investigated were observed. At 75 ppm a marginal but statistically significant increase in the expression of Cyp3a3 (+48.4%; $p \leq 0.05$) was recorded. From 150 ppm, clear dose-related increases were recorded for the expression of the Phase I genes Cyp2b1 (+230.7% at 150 ppm up to +24270% at 1500 ppm) and Cyp3a3 (+159.1% at 150 ppm up to +2051% at 1500 ppm). From 600 ppm, genes coding for Phase II enzymes (i.e. Gsta2, Gstm4, Udpgtr2 and Ephx1) were statistically significantly increased. In addition, Cyp1a1 was statistically significantly increased (+631.1%; $p \leq 0.01$) from this dose level. A marginal, though statistically significant increase (+45%; $p \leq 0.05$) in the marker for apoptosis (Gadd 45b) was also observed. Finally, at 1500 ppm, marginal, though statistically significant increases in those genes associated with cell proliferation/apoptosis (Tacstd 1, Gadd 45b) were recorded.

There were no clear effects on the expression of POR, Cyp4a1, Ephx2 and Rb1. Sult1e1 was only weakly expressed in the liver as evidenced by the variable and high Ct (cycle threshold) values, which for the majority of samples (61/90 control and fluopyram treated liver samples) was greater than or equal to the cut-off limit of 35.

**Table 5.5.4-22**: Mean gene transcript analyses following 3 day treatment with fluopyram

| | **Mean Relative Quantity ± standard deviation of gene transcripts (3 day treatment) (% change compared to control mean values)** | | | | | |
|---|---|---|---|---|---|---|
| **Gene transcripts** | **Control** | **30 ppm** | **75 ppm** | **150 ppm** | **600 ppm** | **1500 ppm** |
| METABOLISM: Phase I | | | | | | |
| POR | **0.87 ± 0.24** | **0.82 ± 0.29 NC** | **0.96 ± 0.37 (+10.3)** | **0.71 ± 0.21 (-18.4)** | **0.95 ± 0.30 (+9.2)** | **0.98 ± 0.39 (+12.6)** |
| Cyp1a1 | **1.35 ± 1.14** | **1.12 ± 0.68 (-17)** | **1.51 ± 1.61 (+11.9)** | **2.3 ± 2.3 (+70.4)** | **9.87 ± 8.29** (+631.1)** | **84.6 ± 51.6** (+6167)** |
| Cyp2b1 | **1.27 ± 1.87** | **0.81 ± 0.56 (-36.2)** | **1.44 ± 0.86 (+13.4)** | **4.2 ± 3.38** (+230.7)** | **63.0 ± 71.2** (+4861)** | **309.5 ± 176.7** (+24270)** |
| Cyp3a3 | **0.93 ± 0.49** | **1.01 ± 0.50 (+8.6)** | **1.38 ± 0.68* (+48.4)** | **2.41 ± 0.89** (+159.1)** | **7.64 ± 2.23** (+721.5)** | **20.0 ± 6.45** (+2051)** |
| Cyp4a1 | **1.46 ± 0.33** | **1.37 ± 0.38 (-6.2)** | **1.51 ± 0.4 (+3.4)** | **1.3 ± 0.4 (-11.0)** | **1.43 ± 0.46 (NC)** | **1.14 ± 0.27 (-21.9)** |
| METABOLISM: Phase II | | | | | | |
| Gsta2 | **0.51 ± 0.45** | **0.58 ± 0.60 (+13.7)** | **0.52 ± 0.34 (NC)** | **0.83 ± 0.54 (+62.7)** | **1.02 ± 0.67** (+100)** | **2.35 ± 1.31** (+360.9)** |
| Gstm4 | **0.83 ± 0.35** | **1.05 ± 0.69 (+26.5)** | **1.24 ± 0.78 (+49.4)** | **1.01 ± 0.99 (+21.7)** | **2.15 ± 1.23** (+159.0)** | **3.86 ± 2.09** (+365)** |
| Udpgtr2 | **1.77 ± 0.93** | **1.68 ± 0.79 (-5.1)** | **2.0 ± 0.89 (+13)** | **3.0 ± 2.13 (+69.5)** | **4.27 ± 2.03** (+141.2)** | **6.66 ± 3.68** (+276.3)** |
| Ephx1 | **0.85 ± 0.24** | **0.91 ± 0.30 (+7.1)** | **0.84 ± 0.31 (NC)** | **1.0 ± 0.36 (+17.6)** | **1.73 ± 0.44** (+103.5)** | **3.12 ± 0.93** (+267.1)** |
| Ephx2 | **1.14 ± 0.31** | **0.92 ± 0.29 (-19.3)** | **0.94 ± 0.21 (-17.5)** | **1.02 ± 0.26 (-10.5)** | **1.18 ± 0.33 (NC)** | **0.95 ± 0.32 (-16.7)** |
| Sult1e1 | **ND** | **ND** | **ND** | **ND** | **ND** | **ND** |
| CELL PROLIFERATION/APOPTOSIS | | | | | | |
| Tacstd1 | **1.19 ± 0.17** | **1.1 ± 0.16 (-7.6)** | **1.1 ± 0.21 (-7.6)** | **1.12 ± 0.23 (-5.9)** | **1.26 ± 0.22 (+5.9)** | **1.66 ± 0.34** (+39.5)** |
| Gadd45b | **0.8 ± 0.26** | **0.8 ± 0.29 (NC)** | **0.84 ± 0.36 (+5)** | **0.9 ± 0.23 (+12.5)** | **1.16 ± 0.45* (+45)** | **1.34 ± 0.57** (+67.5)** |
| Rb1 | **1.01 ± 0.15** | **0.93 ± 0.15 (-7.9)** | **0.96 ± 0.16 (-5)** | **1.05 ± 0.3 (+4)** | **1.04 ± 0.26 (+3)** | **0.82 ± 0.14 * (-18.8)** |

NC: no change; * $p \leq 0.05$; ** $p \leq 0.01$; ND: not detected
In the above table the presented % changes do not always calculate exactly from the presented mean data. This is due to rounding-up differences.

Phenobarbital: The gene expression data indicated that three days treatment with phenobarbital leads to dose-related increased expression of Phase 1 and Phase 2 enzymes. In particular, Cyp2b1, Cyp3a3, both isoforms of glutathione (Gstm4 and Gsta2), Ephx1 and Udpgt were all statistically significantly increased. In contrast there was a statistically significant ($p \leq 0.01$) down-regulation of Cyp4a1 and Ephx2 expression. The marker for cell proliferation (Gadd45b) was also marginally but statistically significantly increased. There were no clear effects on the expression of POR, Cyp1a1 and Rb1. Sult1e1 was only weakly expressed in the liver as evidenced by the variable and high Ct (cycle threshold) values, which for the majority of samples (9/15 phenobarbital treated liver samples) was greater than or equal to the cut-off limit of 35.

**Table 5.5.4-23: Mean gene transcript analyses following 3 day treatment with phenobarbital**

| | Mean Relative Quantity ± standard deviation of gene transcripts (3 day treatment) (% change compared to control mean values) | |
|---|---|---|
| **Gene transcripts** | **Control** | **Phenobarbital (80 mg/kg/day)** |
| METABOLISM: Phase I | | |
| POR | **0.87 ± 0.24** | **0.7 ± 0.24 (-19.5)** |
| Cyp1a1 | **1.35 ± 1.14** | **1.65 ± 2.05 (+22.2)** |
| Cyp2b1 | **1.27 ± 1.87** | **962.8 ± 892.5** (+75711)** |
| Cyp3a3 | **0.93 ± 0.49** | **10.12 ± 7.18** (+988.2)** |
| Cyp4a1 | **1.46 ± 0.33** | **0.52 ± 0.16** (-64.4)** |
| METABOLISM: Phase II | | |
| Gsta2 | **0.51 ± 0.45** | **2.22 ± 1.63** (+335.3)** |
| Gstm4 | **0.83 ± 0.35** | **3.62 ± 3.82* (+336.1)** |
| Udpgtr2 | **1.77 ± 0.93** | **7.83 ± 5.43** (+342. 4)** |
| Ephx1 | **0.85 ± 0.24** | **3.3 ± 2.3** (+288.2)** |
| Ephx2 | **1.14 ± 0.31** | **0.53 ± 0.23** (-53.5)** |
| Sult1e1 | **ND** | **ND** |
| CELL PROLIFERATION/APOPTOSIS | | |
| Tacstd1 | **1.19 ± 0.17** | **1.08 ± 0.18 (-9.2)** |
| Gadd45b | **0.8 ± 0.26** | **1.31 ± 0.67** (+63.75)** |
| Rb1 | **1.01 ± 0.15** | **0.82 ± 0.15** (-18.8)** |

NC: no change; * p≤0.05; ** p≤0.01; ND: not detected

In the above table the presented % changes do not always calculate exactly from the presented mean data. This is due to rounding-up differences.

**Seven day treatment (Subgroup 2):**

Fluopyram:
A similar gene expression profile was observed following 7 days treatment with fluopyram as that observed following 3 days treatment.
At 30 ppm, no significant changes in the expression of any of the genes investigated were observed.
At 75 ppm, a marginal but statistically significant increase in the expression of Cyp3a3 (+95%; p≤0.01) was recorded.
From 150 ppm, clear dose-related increases were recorded for the expression of the Phase I genes Cyp2b1 (+1336% at 150 ppm up to +143300% at 1500 ppm), Cyp3a3 (+262.6% at 150 ppm up to +2756% at 1500 ppm) and Cyp1a1 (+356.2% at 150 ppm up to +22192% at 1500 ppm).
From 600 ppm, genes coding for Phase II enzymes (i.e. Gsta2, Gstm4, Udpgtr2 and Ephx1) were

statistically significantly increased.
Finally, at 1500 ppm, a marginal, though statistically significant increase (+82.5%; p≤0.05) in Gadd45b was recorded. Cyp4a1 (-37%; p≤0.01) and Ephx2 (-39.5%; p≤0.05) were marginally though statistically significantly down-regulated at this dose level.

There were no clear effects on the expression of POR and Rb1. Sult1e1 was only weakly expressed in the liver as evidenced by the variable and high Ct (cycle threshold) values, which for the majority of samples (59/90 control and fluopyram treated liver samples) was greater than the cut-off limit of 35.

**Table 5.5.4-24: Mean gene transcript analyses following 7 day treatment with fluopyram**

| | Mean Relative Quantity ± standard deviation of gene transcripts (7 day treatment) (% change compared to control mean values) | | | | | |
|---|---|---|---|---|---|---|
| **Gene transcripts** | **Control** | **30 ppm** | **75 ppm** | **150 ppm** | **600 ppm** | **1500 ppm** |
| **METABOLISM: Phase I** | | | | | | |
| **POR** | 0.85 ± 0.35 | 0.81 ± 0.29 (-4.7) | 0.81 ± 0.26 (-4.7) | 0.91 ± 0.3 (+7.1) | 1.03 ± 0.42 (+21.2) | 0.99 ± 0.36 (+16.5) |
| **Cyp1a1** | 2.26 ± 1.3 | 3.07 ± 3.87 (+35.8) | 4.0 ± 3.87 (+77) | 10.31 ± 11.01** (+356.2) | 143.7 ± 81.0** (+6258) | 503.8 ± 170.5** (+22192) |
| **Cyp2b1** | 0.95 ± 0.74 | 2.43 ± 1.85 (+155.8) | 2.93 ± 3.87 (+208.4) | 13.64 ± 12.06** (+1336) | 310.2 ± 322.0** (+32553) | 1362.3 ± 1422.2** (+143300) |
| **Cyp3a3** | 0.99 ± 0.59 | 1.46 ± 0.84 (+47.5) | 1.93 ± 0.79** (+95) | 3.59 ± 1.65** (262.6) | 12.32 ± 3.75** (+1144) | 28.27 ± 10.15** (+2756) |
| **Cyp4a1** | 0.73 ± 0.26 | 0.70 ± 0.22 (-4.1) | 0.62 ± 0.14 (-15.1) | 0.64 ± 0.24 (-12.3) | 0.64 ± 0.19 (-12.3) | 0.46 ± 0.11** (-37.0) |
| **METABOLISM: Phase II** | | | | | | |
| **Gsta2** | 2.08 ± 1.7 | 1.37 ± 0.56 (-34.1) | 1.97 ± 1.45 (-5.3) | 2.74 ± 1.78 (+31.7) | 3.6 ± 1.83** (+73.1) | 7.03 ± 4.57** (+238) |
| **Gstm4** | 2.02 ± 1.02 | 2.42 ± 1.76 (+19.8) | 1.81 ± 0.97 (-10.4) | 2.78 ± 1.54 (+37.6) | 4.2 ± 1.82* (+108) | 11.3 ± 19.5** (+459.4) |
| **Udpgtr2** | 2.53 ± 1.28 | 3.58 ± 1.32 (+41.5) | 3.91 ± 1.16** (+54.5) | 3.46 ± 1.5 (+36.8) | 7.33 ± 3.22** (+189.7) | 12.01 ± 4.25** (+374.7) |
| **Ephx1** | 1.2 ± 0.41 | 1.46 ± 0.25 (+21.7) | 1.34 ± 0.32 (+11.7) | 1.2 ± 0.39 (NC) | 2.12 ± 0.69** (+77) | 4.21 ± 2.34** (+250.8) |
| **Ephx2** | 1.19 ± 0.44 | 1.18 ± 0.38 (NC) | 1.09 ± 0.48 (-8.4) | 1.03 ± 0.43 (-13.4) | 0.95 ± 0.38 (-20.2) | 0.72 ± 0.35 * (-39.5) |
| **Sult1e1** | ND | ND | ND | ND | ND | ND |
| **CELL PROLIFERATION/APOPTOSIS** | | | | | | |
| **Tacstd1** | 1.41 ± 0.32 | 1.55 ± 0.37 (+10) | 1.36 ± 0.23 (-3.5) | 1.1 ± 0.26 (-22.0) | 1.38 ± 0.45 (-2.1) | 1.51 ± 0.34 (+7.1) |
| **Gadd45b** | 1.2 ± 0.45 | 1.41 ± 0.43 (+17.5) | 1.13 ± 0.34 (-5.8) | 1.25 ± 0.65 (+4.2) | 1.55 ± 0.53 (+29.2) | 2.19 ± 1.27* (+82.5) |
| **Rb1** | 0.90 ± 0.18 | 0.98 ± 0.15 (+8.9) | 0.89 ± 0.13 (NC) | 0.83 ± 0.17 (-7.8) | 0.87 ± 0.14 (-3.3) | 0.79 ± 0.15 (-12.2) |

NC: no change; * p≤0.05; ** p≤0.01; ND: not detected
In the above table the presented % changes do not always calculate exactly from the presented mean data. This is due to rounding-up differences.

Phenobarbital:
A similar gene expression profile was observed following 7 days treatment with Phenobarbital as that observed following 3 days treatment, although for some genes the expression was more marked after this prolonged treatment. Thus, increased expression of Phase 1 (Cyp2b1; Cyp3a3) and Phase 2 enzymes (Gstm4, Gsta2, Ephx1 and Udpgtr2) was recorded after 7 days with the magnitudes of induction for Cyp2b1 and Cyp3a3 being almost 3-fold and 1.6-fold respectively greater than those observed at the earlier time point. Similarly the induction of Gstm4 was 5.1-fold greater after 7 days

compared to that recorded after 3 days.

As with the earlier time point, there was a statistically significant (p≤0.01) down-regulation of Cyp4a1 and Ephx2 expression and the marker for cell proliferation (Gadd45b) was also marginally but statistically significantly (p≤0.05) increased.

There were no clear effects on the expression of POR, Cyp1a1 and Rb1. Sult1e1 was only weakly expressed in the liver as evidenced by the variable and high Ct (cycle threshold) values, which for the majority of samples (8/15 phenobarbital treated liver samples) was greater than or equal to the cut-off limit of 35.

**Table 5.5.4-25: Mean gene transcript analyses following 7 day treatment with phenobarbital**

| | **Mean Relative Quantity ± standard deviation of gene transcripts (7 day treatment) (% change compared to control mean values)** | |
|---|---|---|
| **Gene transcripts** | **Control** | **Phenobarbital (80 mg/kg/day)** |
| METABOLISM: Phase I | | |
| POR | **0.85 ± 0.35** | **0.66 ± 0.20 (-22.4)** |
| Cyp1a1 | **2.26 ± 1.30** | **1.83 ± 1.24 (-19.0)** |
| Cyp2b1 | **0.95 ± 0.74** | **2776.8 ± 1842.7** (+292195)** |
| Cyp3a3 | **0.99 ± 0.59** | **16.26 ± 10.45** (+1542.4)** |
| Cyp4a1 | **0.73 ± 0.26** | **0.40 ± 0.08** (-45.2)** |
| METABOLISM: Phase II | | |
| Gsta2 | **2.08 ± 1.70** | **4.8 ± 2.3** (+130.8)** |
| Gstm4 | **2.02 ± 1.02** | **18.3 ± 11.8** (+805.9)** |
| Udpgtr2 | **2.53 ± 1.28** | **13.1 ± 7.8** (+418)** |
| Ephx1 | **1.2 ± 0.41** | **4.76 ± 2.43** (+297)** |
| Ephx2 | **1.19 ± 0.44** | **0.81 ± 0.15** (-31.9)** |
| Sult1e1 | **ND** | **ND** |
| CELL PROLIFERATION/APOPTOSIS | | |
| Tacstd1 | **1.41 ± 0.32** | **1.13 ± 0.18** (-19.9)** |
| Gadd45b | **1.2 ± 0.45** | **2.07 ± 0.89** (+72.5)** |
| Rb1 | **0.90 ± 0.18** | **0.71 ± 0.12** (-21.1)** |

NC: no change; * p≤0.05; ** p≤0.01; ND: not detected

In the above table the presented % changes do not always calculate exactly from the presented mean data. This is due to rounding-up differences.

## III. CONCLUSION

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).

| | |
|---|---|
| **Report:** | **KIIA 5.5.4 /24; [redacted]; 2012; M-427431-01** |
| Title: | Fluopyram (AE C 656948): Mechanistic investigations in the liver of female rats following dietary administration |
| Report No & Document No | SA 11104<br>M-427431-01 |
| **Guidelines:** | **No specific guideline** |
| **Deviations:** | **None** |
| **GLP** | **Yes** |

**Executive 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 (batch number: Mix-batch: 08528/0002: a light beige powder, 94.7% w/w) 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≤0.01) and brain weight (+11%; p≤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≤0.01) increased compared to the controls. In addition, statistically significant (p≤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≤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≤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≤0.01), increased expression of the marker for cell proliferation (Gadd 45b) was also observed. Increased activity of BROD (p≤0.01), PROD (p≤ 0.05) and UDPGT-4-nitrophenol (p≤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≤0.01). Statistically significant (p≤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≤0.01), BROD (+88-fold; p≤0.01) and UDPGT-bilirubin (+1.38-fold; p≤0.05) respectively. Enzyme activity of the second isoform of UDPGT (UDPGT-4-nitrophenol) was also statistically significantly increased (+1.81-fold; p≤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.

# I. MATERIALS AND METHODS

## A. MATERIALS:

**1. Test Material:** AE C656948
**Description:** Light beige powder
**Lot/Batch:** Mix-Batch: 08528/0002
**Purity:** 94.7 %
**CAS:** 658066-35-4
**Stability of test compound:** Stable in rodent diet for a period covering the study duration

**2. Vehicle and /or positive control:** Phenobarbital (positive control)
**Description:** White crystalline powder
**Lot/Batch:** Lot No. 09050075
**Purity:** 99 %
**CAS:** 50-06-6
**Stability of test compound:** Stable in 0.5% aqueous solution of methylcellulose for a period covering usage during the study

**3. Test animals:**
**Species**: Rat
**Strain:** Wistar Rj: WI (IOPS HAN) – Female only
**Age:** 10 weeks approximately at start of treatment
**Weight at dosing:** 215 to 261 g
**Acclimation period:** 6-8 days
**Diet:** Certified rodent powdered and irradiated diet A04C-10 P1 from [redacted] ([redacted], [redacted], France), *ad libitum,* except for overnight fasting prior to scheduled necropsy
**Water:** Tap water, *ad libitum*
**Housing:** Animals were caged individually in suspended stainless steel wire mesh cages.
**Environmental conditions –**
**Temperature:** 20 – 24°C
**Humidity:** 40 – 70 %
**Air changes:** Approximately 10-15 changes per hour
**Photoperiod:** Alternating 12-hour light and dark cycles (7 am- 7 pm)

## B. STUDY DESIGN:

**1. In life dates:** Dosing period from May 10 to June 09, 2011, recovery period June 09 to July 08, 2011

**2. Animal assignment and treatment**

Animals were assigned to dose groups using a randomization procedure by weight.

Seven groups of female rats were dosed for at least 28 days with the appropriate compound by the appropriate route of administration.

Six groups were dosed by dietary administration. One group consisted of control animals that received untreated diet and the remaining five groups received fluopyram at the appropriate dietary concentration (30, 75, 150, 600 and 1500 ppm) at a constant level. The dose levels for fluopyram were

set after evaluation of the results from previous studies conducted with the compound and following discussions with the US (EPA) and Canadian (PMRA) authorities. The top dose level of 1500 ppm used in the present study represents the top dose level used in the rat cancer bioassay in which an increase in liver tumors was observed in the females.

The seventh group was dosed by oral gavage with 80 mg/kg/day phenobarbital suspended in 0.5% aqueous methylcellulose 400 using a dosing volume of 5ml/kg bodyweight. The volume administered to each rat was based on the most recent recorded body weight. A dose level of 80 mg/kg/day was chosen for phenobarbital as this dose is known to clearly induce liver cytochrome P-450 activity and cell proliferation in the rat.

Each group consisted of 15 female rats with the exception of the control group, the highest dose fluopyram group and the phenobarbital group, where 15 additional females were fed control or test diet (1500 ppm fluopyram) or were orally dosed (80 mg/kg/day phenobarbital) for at least 28 days and were then allowed one month (at least 28 days) of recovery during which they were maintained on untreated control diet.

All animals were sacrificed in the morning after the last day of treatment or after the last day of the recovery phase.

Animal housing and husbandry were in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals (Public Health Service, National Institute of Health, NIH publication N°86-23, revised 1985) and "Le Guide du Journal Officiel des Communautés Européennes L358, 18 Décembre 1986, N°86/609/CEE du 24 Novembre 1986".

**Table 5.5.4-26: Study design**

| GROUP | TEST SUBSTANCE | DOSE LEVEL | NUMBER OF ANIMALS PER GROUP |
|---|---|---|---|
| FEMALES | | | |
| 1 | Control | 0 | 15 + 15* |
| 2 | Fluopyram | 30 ppm | 15 |
| 3 | | 75 ppm | 15 |
| 4 | | 150 ppm | 15 |
| 5 | | 600 ppm | 15 |
| 6 | | 1500 ppm | 15 + 15* |
| 7 | Phenobarbital | 80 g/kg/day | 15 + 15* |

*Animals allocated to recovery phase

### 3. Diet preparation and analysis

Fluopyram (AE C656948) was incorporated into the diet by dry mixing to provide the required concentrations. There was one preparation for each concentration used in the study. The stability was demonstrated in an earlier study at concentrations of 20 and 10000 ppm for a time which covered the period of usage and storage for the current study. Homogeneity of test substance in diet was verified on the lowest and highest concentrations to demonstrate adequate formulation procedures. The mean value obtained in homogeneity check was used as measured concentration. For the remaining dietary levels, concentration was checked.

Two formulations of phenobarbital were prepared by suspending the test substance (w/w) in a 0.5% aqueous solution of methylcellulose. The stability of phenobarbital at 8 g/l was demonstrated in an earlier study, which covered the period of usage and storage for the current study. Homogeneity of phenobarbital in aqueous methylcellulose was verified to demonstrate adequate formulation formulation procedures. The mean value obtained in homogeneity check was used as measured

concentration.

**4. Statistics**

4.1 Variables analyzed

- Body weight parameters
- Body weight change parameters calculated according to time intervals
- Average food consumption/day parameters calculated according to time intervals
- Terminal body weight, absolute and relative organ weights parameters
- Total cytochrome P450 content and liver enzyme activities
- Cell proliferation parameters
- Gene transcript analysis

4.2 Statistical methods

Mean and standard deviation were calculated for each group.
Group means were compared at the 5% and 1% levels of significance.

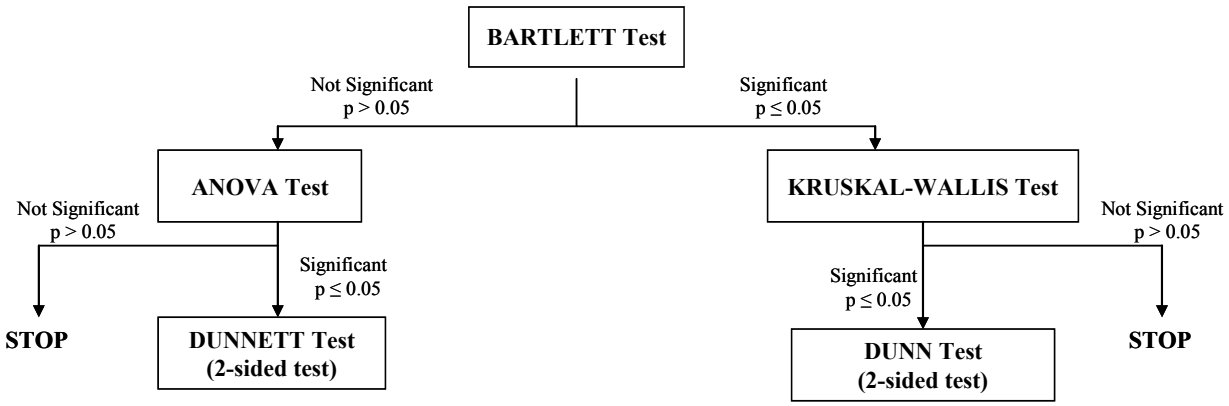
The statistical analyses of the cell proliferation data were carried out by the statistician using the methods described in the appropriate paragraphs.

All other statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics).

*Dosing Phase: comparison between fluopyram groups and control group*

- Body weight change parameters,
- Terminal body weight, absolute and relative organ weight parameters,

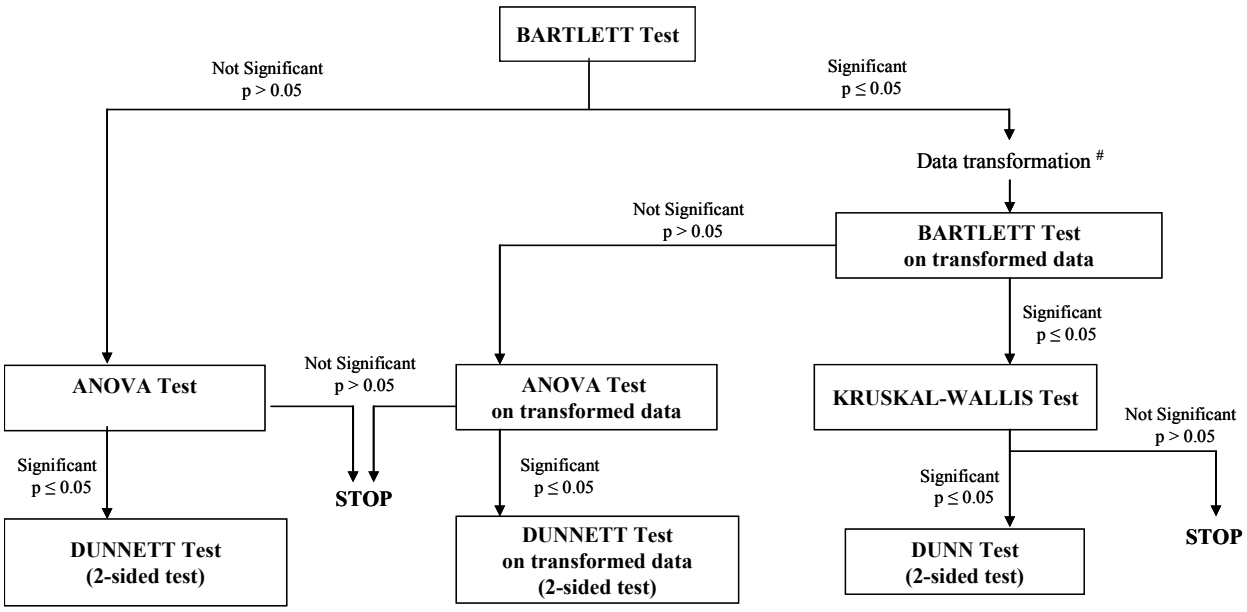
The mean and standard deviation will be calculated for each group and per time period for body weight change parameters.




- Body weight and average food consumption/day parameters
- Total cytochrome P450 content and liver enzyme activities

- Gene transcript analyses

The mean and standard deviation were calculated for each group and per time period for average food consumption/day parameters.




# Data will be transformed using the log transformation for body weight parameters.

- Cell proliferation parameters

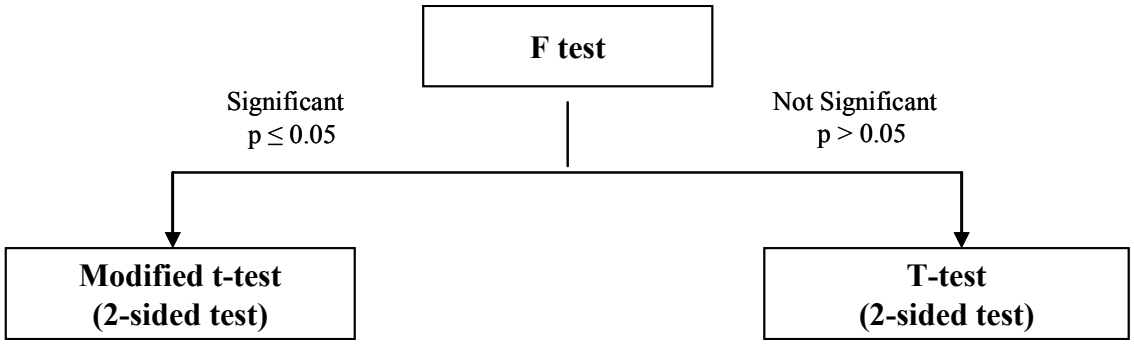
The Levene test was performed to compare the homogeneity of group variances. As the Bartlett test of homogeneity of variances is very sensitive to non-normality of data, the Levene test was preferred.
If the Levene test was not significant (p>0.05), means of the exposed groups were compared to the mean of the control group using the Dunnett test (1-sided).
If the Levene test was significant (p≤0.05), data were transformed using the log transformation.
If the Levene test on log transformed data was not significant (p>0.05), means of the exposed groups were compared to the mean of the control group using the Dunnett test (1-sided) on log transformed data.
If the Levene test was significant (p≤0.05) even after log transformation, means of the exposed groups were compared to the mean of the control group using the Dunn test (1-sided).

If one or more group variance(s) equal 0, means were compared using non-parametric procedures.

*Dosing Phase: comparison between phenobarbital group and control group*
*Recovery Phase: comparison between high dose fluopyram group and control group*
*Recovery Phase: comparison between phenobarbital group and control group*

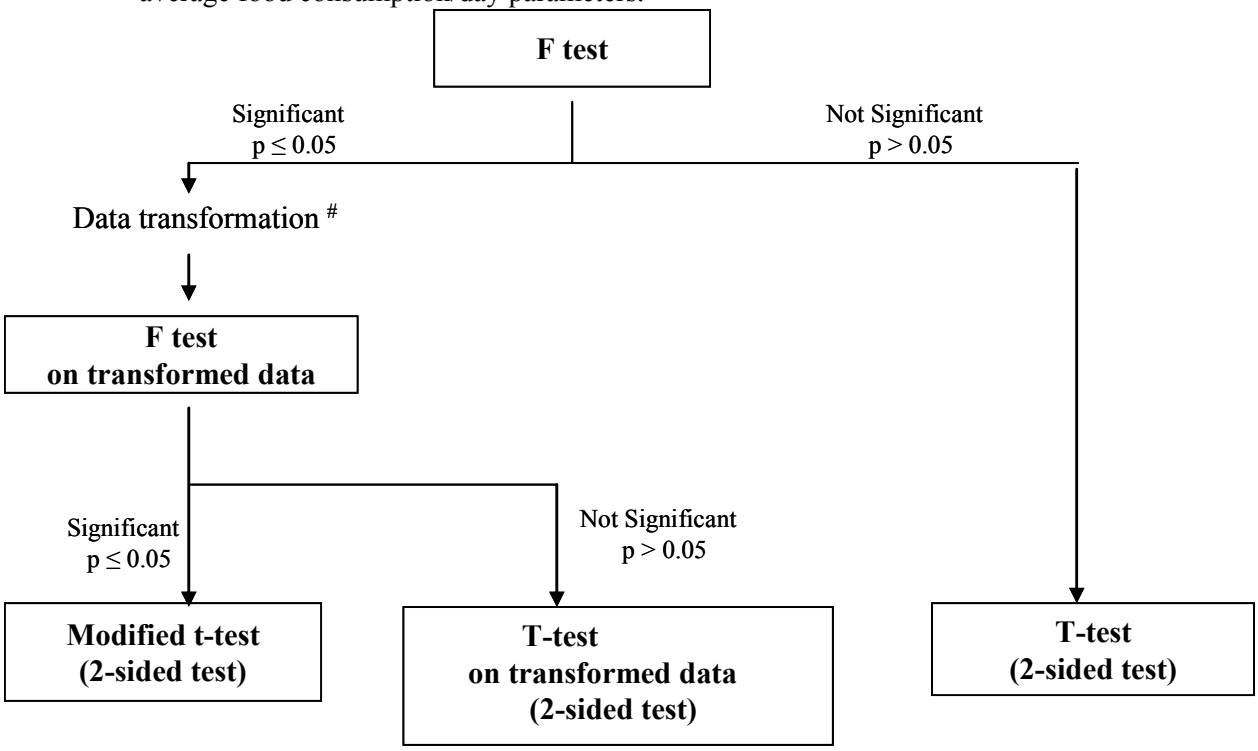
- Body weight change parameters,
- Terminal body weight, absolute and relative organ weight parameters,

  The mean and standard deviation were calculated for each group and per time period for body weight change parameters.




- Body weight and average food consumption/day parameters
- Total cytochrome P450 content and liver enzyme activities
- Gene transcript analyses

The mean and standard deviation were calculated for each group and per time period for average food consumption/day parameters.




# Data will be transformed using the log transformation for body weight parameters.

- Cell proliferation parameters

The F test was performed to compare the homogeneity of group variances.

If the F test was not significant (p>0.05), mean of the test group (high dose fluopyram or phenobarbital) was compared to the mean of the control group using the t-test (1-sided).
If the F test was significant (p≤0.05), data were transformed using the log transformation. If the F test on log transformed data was not significant (p>0.05), mean of the test group (high dose fluopyram or phenobarbital) were compared to the mean of the control group using the t-test (1-sided) on log transformed data.
If the F test was significant (p≤0.05), even after log transformation, mean of the test group were compared to the mean of the control group using the exact Mann-Whitney test (1-sided).

If one or more group variance(s) equal 0, the means were compared using the non-parametric procedures.
Group means were compared at the 5% and 1% levels of significance.

With the exception of those used for the cell proliferation data, all statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics). SAS programs (version 9) were used for the cell proliferation data.

## C. METHODS:

### 1. Observations

The animals were observed twice daily for moribundity and mortality (once daily on weekends or public holidays). Observed clinical signs were recorded at least once daily for all animals and detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility, and duration of any clinical signs were recorded.

### 2. Body weight

Each animal was weighed during the acclimatization period. Body weights were also measured on Day 1 and at least weekly during treatment and the recovery phase. Additionally, diet fasted animals were weighed before scheduled necropsy (terminal body weight).

### 3. Food consumption and compound intake

The weight of food supplied was measured on Day 1 and weekly thereafter. Empty feeder weights were measured weekly. The weight of food supplied to each animal and that remaining at the end of the food consumption period was recorded weekly. Any food spillage was noted. From these records, the weekly mean achieved dosage intake in mg/kg/day for fluopyram for each week and for Weeks 1 to 4 was calculated.

### 4. Sacrifice and pathology

#### 4.1 Necropsy procedure – Organ sampling

All animals were sacrificed by exsanguination under deep anesthesia (inhalation of Isoflurane) in the morning of Day 30 of the treatment phase and Day 29 of the recovery phase. Animals were diet fasted overnight prior to sacrifice.

All animals were necropsied. The necropsy included the examination of the external surfaces, all orifices and all major organs, tissues and body cavities.

Brain and liver were weighed fresh at scheduled sacrifice.
Duodenum and two central sections of the liver taken from the left and medial lobes from each

animal were fixed by immersion in neutral buffered 10% formalin. In addition, a piece of the median and the left lobe of the liver from each animal were collected and flash frozen in liquid nitrogen, were stored at approximately -74°C + 10°C until used for qPCR investigations. At each scheduled sacrifice, the remaining portions of liver from each of 5 randomly selected females per group were weighed and homogenized for microsomal preparations.

## 4.2 Histotechnology

### 4.2.1 Conventional Histopathological examination

Duodenum and the two central sections of the liver were embedded in paraffin wax.
Histological sections, stained with hematoxylin and eosin, were prepared for these two organs for each animal in all groups.

### 4.2.2 Ki67 staining for Cell Proliferation assessment

For each animal in each group a section of a formalin-fixed paraffin-embedded block containing 2 liver samples and one sample of duodenum was prepared. The duodenum was included to serve as a positive control for staining as it has a high rate of cell proliferation. The immunohistochemical reaction included incubation with a monoclonal antibody raised against Ki67, amplification with a secondary biotinylated antibody and a streptavidin-horseradish complex, detection of the complex with diamino-benzidine (DAB) and nuclear counter staining with hematoxylin.

## 4.3 Histopathology

Histopathological examinations were performed on the liver samples from all animals in all groups at both scheduled sacrifices.

## 4.4 Cell Proliferation assessment

The immunohistochemical staining for Ki67 and determination of the labeling index was performed on all surviving animals showing sufficient Ki67 staining (estimated by duodenal Ki67 labeling) to assess cell cycling in the liver. The zonal labeling index, expressed as the number of Ki67-positive hepatocytes per thousand cells, were measured separately on random fields comprising of at least 1000 centrilobular and perilobular cells using an automatic image analysis system. The mean and standard deviation were calculated for each group.

## 4.5 Hepatotoxicity testing

At both scheduled sacrifice times, the remaining portions of the liver from five randomly selected females per group were weighed and homogenized for microsomal preparations in order to determine total cytochrome P-450 content and specific cytochrome P-450 isoenzyme profile (including EROD, BROD and PROD activities) to check the hepatotoxic potential of the test substance. Phase II enzymatic activities were also determined by measuring UDP-glucuronosyltransferase (UDPGT) with bilirubin as substrate. Results were compared to those generated with well known reference compounds.

## 4.6 Total cytochrome P-450 content

Total cytochrome P-450 content in microsomal preparations was determined by spectrophotometry (Cary Win UV version 3.0 (182)) using a reduced CO differential spectrum. A single quantification was performed for each sample.

### 4.7 Enzymatic activities

Cytochrome P-450s and typical inducing agents:

| Family | Enzymatic activity | Activity | Typical inducing agents |
|---|---|---|---|
| CYP 1A1<br>1A2 | } EROD | activation of mutagens and carcinogens | β-naphtoflavone |
| CYP 2<br>2B1<br>2B2 | } PROD | detoxication of drugs and chemicals | phenobarbital |
| 2E | | activation of nitrosamines | isoniazid |
| CYP 3A1 & 3A2 | BROD | detoxication of drugs and chemicals | pregnenolone<br>16 α - carbonitrile<br>phenobarbital |

Specific cytochrome P-450 enzymatic activities were evaluated by spectrofluorimetry (SAFAS SP2000 version 6.10.7.4) using the following substrates:

- ethoxyresorufin (EROD)
- pentoxyresorufin (PROD)
- benzoxyresorufin (BROD)

Ethoxyresorufin is a highly selective substrate for the isoform 1a, the isoform 2b metabolizes preferentially the O-dealkylation of pentoxyresorufin, while the benzoxyresorufin O-debenzylation is mainly metabolized by the isoform 3a. Cytochrome P-450 dependent dealkylation of resorufin derivatives was followed over a period of 2, 5 or 7 minutes at 37°C.

Phase II enzymatic activities were also determined by measuring UDP-glucuronosyltransferase (UDPGT) with 4-nitrophenol as substrate (method adapted from Zakim and Vessey,. The enzymatic kinetic (disappearance of the colored 4-nitrophenol) was followed at 405 nm during 3 min. at 30°C. UDPGT with bilirubin as substrate was also determined using a spectrophotometry method (adapted from Heirwegh et al. consisting in the determination of conjugated bile pigments after its conversion into azo-pigment derivatives. Absorbance was measured at 530 nm. For each isoform of UDPGT three replicates from each sample were assayed.

### 4.8 Quantitative PCR Analyses

#### 4.8.1 Total RNA purification

Total cytoplasmic RNA was isolated from the liver of all surviving individual control and treated animals using RNeasy Midi kits (Qiagen). RNA quality controls were performed based on the ribosomal RNA electrophoretic profiles using a Bioanalyser (Agilent Technologies).

#### 4.8.2 Quantitative PCR

Ten µg of total RNA was used for Reverse transcription (RT) using a High Capacity cDNA Archive kit (Applied Biosystems). The assay was performed in duplicate using Taqman assays (Assay on demand, Applied Biosystems), 1/50 diluted first strand cDNA, Fast Start Universal Probe Master mix (Roche) on an ABI prism 7900 HT machine (Applied Biosystems). For each gene transcript measured, a negative control condition was included in which H2O MQ was used as template instead of first strand cDNA.

The list of Taqman assays used is as follows:

| Gene (Major function) | Abbreviation | Refset ID | Taqman assay ID (Applied Biosystems) |
|---|---|---|---|
| METABOLISM: Phase I | | | |
| P450 (cytochrome)oxidoreductase | POR | NM_031576.1 | Rn00580820_m1 |
| Cytochrome P450 1a1 | Cyp1a1 | NM_012540.2 | Rn00487218_m1 |
| Cytochrome P450 2b1 | Cyp2b1 | 4331248 * | Cyp2b1_tc5 |
| Cytochrome P450 3a3 | Cyp3a3 (Cyp3a23) | NM_013105.2 | Rn01640761_g1 |
| Cytochrome P450 4a1 | Cyp4a1 | NM_175837.1 | Rn00598510_m1 |
| METABOLISM: Phase II | | | |
| Glutathione S-transferase A2 | Gsta2 | NM_017013.4 | Rn00566636_m1 |
| Glutathione S-transferase mu3 | Gstm4 | NM_020540.1 | Rn01789233_m1 |
| UDP glucoronosyltransferase 2 family, polypeptide B1 | Udpgtr2 | NM_173295.1 | Rn00756519_m1 |
| Epoxide hydrolase 1, microsomal | Ephx1 | NM_012844.2 | Rn00563349_m1 |
| Epoxide hydrolase 2, cytoplasmic | Ephx2 | NM_022936.1 | Rn00576023_m1 |
| Sulfotranferase family 1E, member 1 | Sult1e1 | NM_012883.1 | Rn00820646_g1 |
| CELL PROLIFERATION/APOPTOSIS | | | |
| Epithelial cell adhesion molecule | Tacstd1 | NM_138541.1 | Rn01473202_m1 |
| Growth arrest and DNA-damage-inducible 45 beta | Gadd45b | NM_001008321.1 | Rn01452530_g1 |
| Retinoblastoma 1 | Rb1 | XM_344434.3 | Rn01753308_m1 |

*: Transcript made to order

Beta-microglobulin (B2m; Refset ID: NM_012512.1; Taqman assay ID: Rn00560865_m1) was selected as reference gene for the quantitative calculations of transcripts in the liver. The relative quantity (RQ) value of each test transcript was calculated using the following formula:

$\Delta\Delta Ct = (Ct_{test} - Ct_{B2m})_{treated} - (Ct_{test} - Ct_{B2m})_{control}$

$RQ = 2^{-\Delta\Delta Ct}$

where Ct is the threshold cycle at which PCR amplification started to be significantly different from the background signal. As a Ct of ≥35 indicates that a gene is poorly expressed in the tissue investigated, any subsequent RQ data generated from such a Ct are considered as non-relevant due to an increased risk of contamination.

## II. RESULTS AND DISCUSSION

### A. OBSERVATIONS:

Mortality:

There was no mortality in any group during the course of the study.

Clinical signs:

**Treatment phase:**

Fluopyram: Four females in three different treatment groups had ocular discharge from Week 3 of treatment. One of the four females also had red conjunctiva in the same eye as the discharge.

Phenobarbital: All females displayed reduced motor activity starting from Week 2 of treatment. In addition, three females had ocular discharge and/or lacrimation in either one eye or both eyes starting from Week 2 of treatment. Other clinical signs included increased salivation for two females during the final week of treatment and soiling around the mouth or the head.

**Recovery phase:**

The few clinical signs (described below) recorded during the recovery phase were considered not to be treatment-related.

Fluopyram: Two females at 1500 ppm exhibited soiled fur around the head region during Week 2 of the recovery phase.

Phenobarbital: One female exhibited soiled fur around the nose during Week 4 of the recovery phase and one female had ocular discharge from one eye during Week 2 of the recovery phase.

### B. BODY WEIGHT AND BODY WEIGHT GAIN:

**Treatment phase:**

Fluopyram: There were no relevant treatment-related changes in any of the body weight parameters compared to the controls during the treatment phase.

Phenobarbital: There were no relevant changes in any of the body weight parameters compared to the controls during the treatment phase.

**Recovery phase:**

Fluopyram: There were no relevant changes in any of the body weight parameters during the recovery phase compared to the controls.

Phenobarbital: Between Days 1 and 8 of the recovery phase, there was a mean absolute body weight loss compared to the control group (-2g compared to +8g in the control group; -125%, $p \leq 0.01$). Between Days 8 and 15 of the recovery phase there was a reduced mean absolute body weight gain compared to the control group (5g compared to 10g in the control group; -50%, not statistically significant). Thereafter, the mean absolute body weight gains were comparable to the controls.

## C. FOOD CONSUMPTION AND COMPOUND INTAKE:

**Treatment phase:**

Fluopyram: Food consumption was statistically significantly reduced at 1500 ppm during week 3 ($p \leq 0.05$) and week 4 ($p \leq 0.01$) of treatment. All other changes were considered non-relevant.

Phenobarbital: Food consumption was increased by up to 12% compared to the controls for all periods during the treatment phase. This increase was statistically significant for the first ($p \leq 0.05$), second ($p \leq 0.01$) and fourth ($p \leq 0.05$) week of treatment.

**Recovery phase:**

Fluopyram: A marginal, though statistically significant reduction ($p \leq 0.05$) in food consumption was recorded during the second and third week of the recovery phase.

Phenobarbital: A significant reduction ($p \leq 0.01$) in food consumption was recorded for the first week of the recovery phase, which could be attributed to two females with an apparent reduced food intake. Thereafter, food intake was comparable to the controls.

**Table 5.5.4-27: Mean achieved intake of fluopyram**

| Diet Concentration (ppm) of fluopyram | Mean achieved dietary intake of fluopyram (mg/kg/day) for weeks 1-4 |
|---|---|
| 30 | 2.2 |
| 75 | 5.6 |
| 150 | 11.3 |
| 600 | 44.5 |
| 1500 | 111.4 |

## D. SACRIFICE AND PATHOLOGY

### 1. Terminal body weight and organ weight

**Treatment phase:**

Fluopyram: There was no change in mean terminal body weight in treated animals when compared to the controls.

At 600 and 1500 ppm, mean absolute and relative liver weight were statistically significantly higher when compared to controls.

At 150 ppm, mean liver to body weight ratio and mean liver to brain weight ratio were statistically significantly higher when compared to controls.
These changes were considered to be treatment-related.

**Table 5.5.4-28: Mean absolute and relative liver weight changes following 28 days treatment with fluopyram**

| MEAN LIVER WEIGHT ±SD AT SCHEDULED SACRIFICE (% CHANGE WHEN COMPARED TO CONTROLS) | | | | | | |
|---|---|---|---|---|---|---|
| Dose-level (ppm) | Fluopyram | | | | | |
| | 0 | 30 | 75 | 150 | 600 | 1500 |
| Absolute liver weight (g) | 6.14±0.53 | 6.10±0.26 (-1%) | 6.48±0.46 (+6%) | 6.64±0.77 (+8%) | 6.78±0.49* (+10%) | 7.90±0.53** (+29%) |
| Liver to body weight ratio (%) | 2.38±0.16 | 2.42±0.11 (+2%) | 2.49±0.13 (+5%) | 2.55±0.11** (+7%) | 2.69±0.15** (+13%) | 3.16±0.14** (+33%) |
| Liver to brain weight ratio (%) | 319.44 ±31.96 | 319.41 ±18.44 (0%) | 342.46 ±24.12 (+7%) | 353.35 ±39.95* (+11%) | 368.06 ±28.60** (+15%) | 424.55 ±30.85** (+33%) |

*: p≤0.05, **: p≤0.01

The few other organ weight changes were considered to be incidental.

Phenobarbital: There was no change in mean terminal body weight in treated animals when compared to the controls.
Mean absolute and relative liver weight were statistically significantly higher when compared to controls following phenobarbital treatment.

**Table 5.5.4-29: Mean absolute and relative liver weight changes following 28 days treatment with phenobarbital**

| MEAN LIVER WEIGHT ±SD AT SCHEDULED SACRIFICE (% CHANGE WHEN COMPARED TO CONTROLS) | | |
|---|---|---|
| Dose-level | Phenobarbital | |
| | 0 ppm | 80 mg/kg |
| Absolute liver weight (g) | 6.14 ± 0.53 | 7.48 ± 1.01** (+22%) |
| Liver to body weight ratio (%) | 2.382 ± 0.160 | 2.936 ± 0.352** (+23%) |
| Liver to brain weight ratio (%) | 319.44 ± 31.96 | 412.05 ± 51.53** (+29%) |

**: p≤0.01

The few other organ weight changes were considered to be incidental.

**Recovery phase:**

Fluopyram: There was no change in mean terminal body weight in treated animals when compared to the controls.

The few organ weight changes were considered to be incidental.

Phenobarbital: There was no change in mean terminal body weight in treated animals when compared to the controls.

The few organ weight changes were considered to be incidental.

## 2. Gross pathology

**Treatment phase:**

Fluopyram: Enlarged liver was observed starting from 75 ppm and this observation could be correlated with microscopic findings starting from 600 ppm.

Other changes were considered as incidental and not treatment-related (no correlation with microscopic findings).

**Table 5.5.4-30: Macroscopic changes in the liver following 28 days treatment with fluopyram**

| INCIDENCE OF MACROSCOPIC CHANGES IN THE LIVER, SCHEDULED SACRIFICE | | | | | | |
|---|---|---|---|---|---|---|
| **Dose-level (ppm)** | **Fluopyram** | | | | | |
| | **0** | **30** | **75** | **150** | **600** | **1500** |
| **Enlarged** | **0/15** | **0/15** | **3/15** | **5/15** | **4/15** | **14/15** |

Phenobarbital: Enlarged livers were noted in 10/15 animals treated with phenobarbital.

Other changes were considered as incidental and not treatment-related.

**Recovery phase:**

Fluopyram: All changes were considered as incidental and not treatment-related.

Phenobarbital: All changes were considered as incidental and not treatment-related.

## 3. Microscopic pathology

**Treatment phase:**

Fluopyram: Higher incidences of hepatocellular hypertrophy compared to the controls were noted at 600 and 1500 ppm and were considered to be treatment-related.

Phenobarbital: Higher incidences of hepatocellular hypertrophy and increased number of mitoses were noted and were considered to be treatment-related.

**Table 5.5.4-31: Microscopic changes in the liver following 28 days treatment with fluopyram or phenobarbital**

| INCIDENCE AND SEVERITY OF MICROSCOPIC CHANGES IN THE LIVER, ALL ANIMALS OF THE TERMINAL SACRIFICE | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Dose level of Fluopyram (ppm)** | **0** | **30** | **75** | **150** | **600** | **1500** | **PB (80 mg/kg)** |
| **Number of examined animals** | **15** | **15** | **15** | **15** | **15** | **15** | **15** |
| **Hepatocellular hypertrophy: centrilobular to panlobular** | | | | | | | |
| **Minimal** | **0** | **0** | **0** | **0** | **6** | **7** | **6** |
| **Slight** | **0** | **0** | **0** | **0** | **0** | **6** | **5** |
| **Moderate** | **0** | **0** | **0** | **0** | **0** | **1** | **1** |
| **Total** | **0** | **0** | **0** | **0** | **6** | **14** | **12** |
| **Increased number of mitoses** | | | | | | | |
| **Present** | **0** | **0** | **0** | **0** | **0** | **0** | **1** |
| **Total** | **0** | **0** | **0** | **0** | **0** | **0** | **1** |

**Recovery phase:**

Fluopyram: All changes were considered as incidental and not treatment-related.

Phenobarbital: All changes were considered as incidental and not treatment-related.

## 4. Cell proliferation

**Treatment phase:**

Fluopyram: Centrilobular and global (referred to as "Total" in tables below) proliferation indexes were statistically significantly higher from 150 ppm when compared to controls. Periportal proliferation index was also significantly higher at 1500 ppm when compared to controls. Centrilobular proliferation was statistically significantly increased at 75 ppm.

Phenobarbital: Centrilobular and global proliferation indexes were statistically significantly higher when compared to controls.

**Table 5.5.4-32: Mean cell proliferation index following 28 days treatment with fluopyram or phenobarbital**

| | | Mean ± SD[A] (% change compared to control mean values) | | |
|---|---|---|---|---|
| **Compound** | **Dose** | **Centrilobular** | **Periportal** | **Total** |
| **Control** | **0** | **4.93 ± 3.11** | **8.37 ± 4.75** | **6.65 ± 3.19** |
| **Fluopyram** | **30 ppm** | **4.23 ± 2.42 (-14%)** | **7.62 ± 3.66 (-9%)** | **5.93 ± 2.82 (-11%)** |
| | **75 ppm** | **7.23 ± 3.55* (+47%)** | **8.51 ± 3.86 (+2%)** | **7.87 ± 2.65 (+18%)** |
| | **150 ppm** | **10.16 ± 3.86** (+106%)** | **12.51 ± 3.97* (+49%)** | **11.33 ± 3.30** (+70%)** |
| | **600 ppm** | **10.14 ± 5.27** (+106%)** | **12.10 ± 8.36 (+45%)** | **11.12 ± 6.50** (+67%)** |
| | **1500 ppm** | **15.54 ± 7.33** (+215%)** | **22.80 ± 10.49** (+172%)** | **19.17 ± 7.20** (+188%)** |
| **Phenobarbital** | **80 mg/kg** | **21.46 ± 17.90** (+335%)** | **10.28 ± 7.24 (+23%)** | **15.87 ± 11.74** (+139%)** |

A: n = 15 females/group for cell proliferation determinations; *: p≤0.05; **: p≤0.01. Differences in data between table above and in Appendix K are due to rounding-up.

**Recovery phase:**

Fluopyram: Centrilobular and global proliferation indexes were statistically significantly higher when compared to the control group.

Phenobarbital: Centrilobular, periportal and global proliferation indexes were significantly higher when compared to the control group.

**Table 5.5.4-33: Mean cell proliferation index following Recovery Phase**

| | Mean ± SD[A] | | |
|---|---|---|---|
| | **Centrilobular** | **Periportal** | **Total** |
| **Control** | **4.59 ± 2.44** | **8.25 ± 4.60** | **6.42 ± 3.29** |
| **1500 ppm** | **8.30 ± 3.75** (+81%)** | **11.12 ± 6.87 (+35%)** | **9.71 ± 4.77* (+51%)** |
| **PB (80 mg/kg)** | **6.91 ± 3.44* (+51%)** | **16.92 ± 9.83** (+105%)** | **11.92 ± 6.47** (+86%)** |

A: n = 15 females/group for cell proliferation determinations with the exception of the control group where n=14 instead of 15; *: p≤0.05; **: p≤0.01. Differences in data between table above and in Appendix K are due **to** rounding-up.

## 5. Hepatotoxicity testing

**Treatment phase:**

Fluopyram: A marginal, though not statistically significant increase in total P450 content was recorded at 1500 ppm. No statistically significant changes in enzyme activity were observed at 30 ppm and 75 ppm. Statistically significant increases in UDPGT-bilirubin (p≤0.01) were observed from 150 ppm. EROD activity was also statistically significantly increased from this dose level, however the increases (up to 96% at 1500 ppm) were considered marginal in comparison with the positive control, beta-naphthoflavone, which induced EROD by 800% compared to controls. BROD (p≤0.01), PROD (p≤0.05) and UDPGT-4-nitrophenol (p≤0.01) were statistically significantly increased from 600 ppm.

**Table 5.5.4-34: Mean cytochrome P-450 content and enzymatic activities in the liver following 28 days treatment with fluopyram or phenobarbital**

| Fluopyram dose | Mean Fold Change Relative to Controls | | | | | |
|---|---|---|---|---|---|---|
| | Total P450 Content | EROD | BROD | PROD | UDPGT-4-nitrophenol | UDPGT-bilirubin[A] |
| 30 ppm | 0.94 | 1.13 | 1.24 | 0.92 | 0.82 | 1.09 |
| 75 ppm | 0.97 | 1.08 | 1.44 | 1.27 | 0.95 | 1.21 |
| 150 ppm | 1.24 | 1.34** | 2.89 | 1.53 | 1.08 | 1.57** |
| 600 ppm | 1.05 | 1.31* | 9.12** | 1.87* | 1.83** | 2.14** |
| 1500 ppm | 1.42 | 1.96** | 39.09** | 4.76** | 3.21** | 2.76** |
| Phenobarbital 80 mg/kg | 1.51** | 1.07 | 87.4** | 8.44* | 1.81** | 1.38* |

**A: n=4 instead of 5 for the control group due to one outlier animal. *: p≤0.05; **: p≤0.01**

**Recovery phase:**

Fluopyram: EROD (p≤0.05), BROD (p≤0.05), PROD (p≤0.01) and UDPGT-bilirubin (p≤0.05) activity were statistically significantly increased following 1 month on control diet; however the increases were much lower than those recorded immediately following treatment.

Phenobarbital: PROD and UDPGT-bilirubin activity were statistically significantly increased (p≤0.01) following 1 month recovery. The increase in PROD activity was much lower than that recorded immediately after treatment; however UDPGT-bilirubin activity was marginally increased compared to immediately following treatment (1.56-fold following recovery compared to 1.38-fold following treatment). EROD was also significantly increased (p≤0.01) following the recovery phase despite there being no activity immediately following treatment. The increase (27%) was considered marginal in comparison with the positive control, beta-naphthoflavone, which induced EROD by 800% compared to controls.

**Table 5.5.4-35: Mean cytochrome P-450 content and enzymatic activities in the liver following Recovery Phase**

| Dose | Mean Fold change Relative to Controls | | | | | |
|---|---|---|---|---|---|---|
| | Total P450 Content | EROD | BROD | PROD | UDPGT-4-nitrophenol | UDPGT-bilirubin |
| **Fluopram 1500 ppm** | **1.03** | **1.21*** | **1.47*** | **1.54**** | **1.01** | **1.39*** |
| **Phenobarbital 80 mg/kg/day** | **1.01** | **1.28**** | **0.85** | **1.93**** | **0.98** | **1.56**** |

***: p≤0.05; **: p≤0.01**

## 6. Gene Transcript Analyses

**Treatment phase:**

Fluopyram: At 30 ppm, a clear and statistically significant (p≤0.01) dose-related increase in the expression of Cyp3a3 was recorded (+81% at 30 ppm up to +4943% at 1500 ppm).

From 150 ppm, clear dose-related and statistically significant (p≤0.01) increases were recorded for the expression of the Phase I genes Cyp2b1 (+990% at 150 ppm up to +154275% at 1500 ppm) and Cyp1a1 (+711% at 150 ppm up to +35372% at 1500 ppm). In addition, a statistically significant (p≤0.01) increase was recorded for the expression of genes coding for the Phase II enzymes Gstm4 (+136% at 150 ppm up to 1289% at 1500 ppm) and Udpgtr2 (+64% at 150 ppm up to +304% at 1500 ppm).

From 600 ppm, genes coding for additional Phase II enzymes were statistically significantly increased. Specifically, Gsta2 expression was increased by 53% (p≤0.05) at this dose and Ephx1 expression was increased by +116% (p≤0.01). A marginal, though statistically significant (+75%; p≤0.01), increased expression of the marker for cell proliferation (Gadd 45b) was also observed.

Finally, the expression of Ephx2 demonstrated a weak but dose-related decrease (-3%, -10%, -13%, -27% at 30, 75, 150 and 600 ppm respectively), which was statistically significant at 1500 ppm (-43%; p≤0.01).

There were no clear effects on the expression of POR, Cyp4a1, Tacstd1 and Rb1. Sult1e1 was only weakly expressed in the liver as evidenced by the variable and high Ct (cycle threshold) values, which was greater than or equal to the cut-off limit of 35 for the majority of samples (59/90 control and fluopyram treated liver samples).

**Table 5.5.4-36: Mean gene transcript analyses following 28 days treatment with fluopyram**

| | Mean Relative Quantity ± standard deviation of gene transcripts (28 day treatment) (% change compared to control mean values) | | | | | |
|---|---|---|---|---|---|---|
| **Gene transcripts** | **Control** | **30 ppm** | **75 ppm** | **150 ppm** | **600 ppm** | **1500 ppm** |
| **METABOLISM: Phase I** | | | | | | |
| **POR** | 0.78 ± 0.34 | 0.70 ± 0.29 (-10%) | 0.67 ± 0.29 (-14%) | 0.77 ± 0.27 (NC) | 0.81 ± 0.24 (+4%) | 0.89 ± 0.30 (+14%) |
| **Cyp1a1** | 1.06 ± 0.70 | 1.87 ± 1.25 (+76%) | 2.43 ± 1.48 (+129%) | 8.60 ± 7.02** (+711%) | 107.0 ± 28.03** (+999%) | 376.0 ± 130.43** (+35372%) |
| **Cyp2b1** | 1.26 ± 1.15 | 3.38 ± 7.17 (+168%) | 2.09 ± 1.91 (+66%) | 13.74 ± 10.57** (+990%) | 267.69 ± 193.39** (+21145%) | 1945.13 ± 1518.28** (+154275%) |
| **Cyp3a3** | 1.66 ± 0.65 | 3.01 ± 1.34** (+81%) | 6.19 ± 2.66** (+273%) | 8.72 ± 2.69** (+425%) | 28.37 ± 7.22** (1609%) | 83.72 ± 27.02** (+4943%) |
| **Cyp4a1** | 0.78 ± 0.30 | 0.65 ± 0.25 (-17%) | 0.69 ± 0.20 (-12%) | 0.77 ± 0.16 (NC) | 0.63 ± 0.18 (-19%) | 0.55 ± 0.08 (-30%) |
| **METABOLISM: Phase II** | | | | | | |
| **Gsta2** | 3.62 ± 2.63 | 3.76 ± 2.19 (+4%) | 3.43 ± 2.34 (-5%) | 3.28 ± 2.37 (-9%) | 5.54 ± 2.54* (+53%) | 10.85 ± 6.21** (+200%) |
| **Gstm4** | 0.56 ± 0.39 | 0.60 ± 0.36 (+7%) | 0.92 ± 0.62 (+64%) | 1.32 ± 0.48** (+136%) | 3.42 ± 2.82** (+511%) | 7.78 ± 6.64** (+1289%) |
| **Udpgtr2** | 0.90 ± 0.40 | 1.08 ± 0.40 (+20%) | 1.04 ± 0.58 (+16%) | 1.48 ± 0.51** (+64%) | 2.29 ± 0.92** (+154%) | 3.64 ± 1.51** (+304%) |
| **Ephx1** | 1.06 ± 0.22 | 1.34 ± 0.60 (+26%) | 1.18 ± 0.31 (+11%) | 1.49 ± 0.35 (+41%) | 2.29 ± 0.58** (+116%) | 4.49 ± 2.04** (+324%) |
| **Ephx2** | 1.73 ± 0.64 | 1.67 ± 0.64 (-3%) | 1.55 ± 0.74 (-10%) | 1.51 ± 0.66 (-13%) | 1.27 ± 0.54 (-27%) | 0.98 ± 0.29** (-43%) |
| **Sult1e1** | ND | ND | ND | ND | ND | ND |
| **CELL PROLIFERATION/APOPTOSIS** | | | | | | |
| **Tacstd1** | 1.09 ± 0.31 | 1.30 ± 0.38 (+19%) | 1.16 ± 0.23 (+6%) | 1.35 ± 0.25 (+24%) | 1.23 ± 0.28 (+13%) | 1.35 ± 0.32 (+24%) |
| **Gadd45b** | 0.79 ± 0.23 | 0.85 ± 0.44 (+8%) | 0.76 ± 0.20 (-4%) | 0.75 ± 0.20 (-5%) | 1.38 ± 0.49** (+75%) | 1.75 ± 0.89** (+122%) |
| **Rb1** | 1.15 ± 0.26 | 1.14 ± 0.19 (NC) | 1.10 ± 0.19 (-4%) | 1.21 ± 0.18 (+5%) | 1.16 ± 0.19 (NC) | 1.16 ± 0.23 (NC) |

NC: no change; *: p≤0.05; **: p≤0.01; ND: considered as not detected due to the variable and high Ct values, which were greater than or equal to the cut-off limit of 35 for the majority of samples (59/90 control and fluopyram treated liver samples). In the above table the presented % changes do not always calculate exactly from the presented mean data. This is due to rounding-up differences.

Phenobarbital: The gene expression data indicated that treatment with phenobarbital leads to dose-related increased expression of Phase I and Phase II enzymes. In particular, Cyp2b1, Cyp3a3, both isoforms of glutathione (Gstm4 and Gsta2), Ephx1 and Udpgt were all statistically significantly increased. In contrast there was a statistically significant ($p \leq 0.01$) down-regulation of Cyp4a1 and Ephx2 expression.
The marker for cell proliferation (Gadd45b) was also statistically significantly increased.
There were no clear effects on the expression of POR, Cyp1a1 and Rb1. Sult1e1 was only weakly expressed in the liver as evidenced by the variable and high Ct (cycle threshold) values, which were greater than or equal to the cut-off limit of 35 for the majority of samples (8/15 phenobarbital treated liver samples).

**Table 5.5.4-37: Mean gene transcript analyses following 28 days treatment with phenobarbital**

| | Mean Relative Quantity ± standard deviation of gene transcripts (28 day treatment) (% change compared to control mean values) | |
|---|---|---|
| **Gene transcripts** | **Control** | **Phenobarbital (80 mg/kg/day)** |
| **METABOLISM: Phase I** | | |
| **POR** | **0.78 ± 0.34** | **0.60 ± 0.25 (-23%)** |
| **Cyp1a1** | **1.06 ± 0.70** | **0.80 ± 0.41 (-25%)** |
| **Cyp2b1** | **1.26 ± 1.15** | **2930.45 ± 2578.47** (+232475%)** |
| **Cyp3a3** | **1.66 ± 0.65** | **54.21 ± 43.44** (+32%)** |
| **Cyp4a1** | **0.78 ± 0.30** | **0.41 ± 0.08** (-47%)** |
| **METABOLISM: Phase II** | | |
| **Gsta2** | **3.62 ± 2.63** | **6.33 ± 4.30* (+75%)** |
| **Gstm4** | **0.56 ± 0.39** | **13.85 ± 11.39** (+2373%)** |
| **Udpgtr2** | **0.90 ± 0.40** | **3.92 ± 2.66** (+336%)** |
| **Ephx1** | **1.06 ± 0.22** | **3.67 ± 1.77** (+246%)** |
| **Ephx2** | **1.73 ± 0.64** | **0.95 ± 0.27** (-45%)** |
| **Sult1e1** | **ND** | **ND** |
| **CELL PROLIFERATION/APOPTOSIS** | | |
| **Tacstd1** | **1.09 ± 0.31** | **1.16 ± 0.42 (+6%)** |
| **Gadd45b** | **0.79 ± 0.23** | **1.33 ± 0.59** (+68%)** |
| **Rb1** | **1.15 ± 0.26** | **1.28 ± 0.23 (+11%)** |

NC: no change; *: $p \leq 0.05$; **: $p \leq 0.01$; ND: considered as not detected due to the variable and high Ct values, which were greater than or equal to the cut-off limit of 35 for the majority of samples (19/30 for controls and phenobarbital). In the above table the presented % changes do not always calculate exactly from the presented mean data. This is due to rounding-up differences.

**Recovery phase:**

Fluopyram: The expression of genes coding for the Phase I enzymes Cyp1a1 (+83%), Cyp2b1 (+70%) and Cyp3a3 (+157%; p≤0.01) and the Phase II enzyme Gstm4 (+73%; p≤0.05) were still increased compared to the controls. Gadd45b (+42%; p≤0.01) and Rb1 (+13%; p≤0.05) were also statistically significantly increased compared to the controls. In all cases however; the % changes were much lower than those recorded immediately following treatment.

Sult1e1 was only weakly expressed in the liver as evidenced by the variable and high Ct (cycle threshold) values, which were greater than the cut-off limit of 35 for the majority of samples (25/30 control and fluopyram treated liver samples).

**Table 5.5.4-38: Mean gene transcript analyses following Recovery Phase in fluopyram treated group**

| | Mean Relative Quantity ± standard deviation of gene transcripts (Recovery Phase) (% change compared to control mean values) | |
|---|---|---|
| **Gene transcripts** | **Control** | **1500 ppm** |
| **METABOLISM: Phase I** | | |
| **POR** | **0.54 ± 0.24** | **0.62 ± 0.18 (+15%)** |
| **Cyp1a1** | **0.72 ± 0.23** | **1.32 ± 1.11 (+83%)** |
| **Cyp2b1** | **0.30 ± 0.24** | **0.51 ± 0.36 (+70%)** |
| **Cyp3a3** | **2.67 ± 1.61** | **6.86 ± 3.75** (+157%)** |
| **Cyp4a1** | **0.68 ± 0.29** | **0.76 ± 0.14 (+12%)** |
| **METABOLISM: Phase II** | | |
| **Gsta2** | **1.55 ± 1.37** | **1.67 ± 1.12 (+8%)** |
| **Gstm4** | **0.74 ± 0.48** | **1.28 ± 0.95* (+73%)** |
| **Udpgtr2** | **0.83 ± 0.33** | **0.65 ± 0.25 (-22%)** |
| **Ephx1** | **0.80 ± 0.19** | **0.86 ± 0.31 (+8%)** |
| **Ephx2** | **0.85 ± 0.26** | **1.11 ± 0.29* (+31%)** |
| **Sult1e1** | **ND** | **ND** |
| **CELL PROLIFERATION/APOPTOSIS** | | |
| **Tacstd1** | **1.21 ± 0.29** | **1.31 ± 0.27 (+8%)** |
| **Gadd45b** | **1.38 ± 0.42** | **1.96 ± 0.57** (+42%)** |
| **Rb1** | **1.02 ± 0.14** | **1.15 ± 0.17* (+13%)** |

NC: no change; *: p≤0.05; **: p≤0.01; ND: considered as not detected due to the variable and high Ct values, which were greater than or equal to the cut-off limit of 35 for the majority of samples (25/30 for controls and fluopyram). In the above table the presented % changes do not always calculate exactly from the presented mean data. This is due to rounding-up differences.

Phenobarbital: The expression of genes coding for the Phase I enzymes Cyp2b1 (+107%; p≤0.01), Cyp3a3 (+179%; p≤0.01) and Cyp4a1 (+46%; p≤0.01) and the Phase II enzyme Gstm4 (+181%; p≤0.01) were still increased compared to the controls. Ephx1 (+21%; p≤0.05) was also increased following the recovery phase. However, with the exception of Cyp4a1 and Ephx1, which were not increased immediately following treatment, the % changes were much lower than those recorded immediately following treatment.
Sult1e1 was only weakly expressed in the liver as evidenced by the variable and high Ct (cycle threshold) values, which were greater than or equal to the cut-off limit of 35 for the majority of samples (24/30 control and phenobarbital treated liver samples).

**Table 5.5.4-39: Mean gene transcript analyses following Recovery Phase in phenobarbital treated group**

| | Mean Relative Quantity ± standard deviation of gene transcripts (Recovery Phase) (% change compared to control mean values) | |
|---|---|---|
| **Gene transcripts** | **Control** | **Phenobarbital (80 mg/kg/day)** |
| **METABOLISM: Phase I** | | |
| **POR** | **0.54 ± 0.24** | **0.76 ± 0.23* (+41%)** |
| **Cyp1a1** | **0.72 ± 0.23** | **0.87 ± 0.45 (21%)** |
| **Cyp2b1** | **0.30 ± 0.24** | **0.62 ± 0.46** (+107%)** |
| **Cyp3a3** | **2.67 ± 1.61** | **7.46 ± 5.02** (+179%)** |
| **Cyp4a1** | **0.68 ± 0.29** | **0.99 ± 0.29** (+46%)** |
| **METABOLISM: Phase II** | | |
| **Gsta2** | **1.55 ± 1.37** | **1.98 ± 2.11 (+28%)** |
| **Gstm4** | **0.74 ± 0.48** | **2.08 ± 1.22** (+181%)** |
| **Udpgtr2** | **0.83 ± 0.33** | **0.69 ± 0.38 (-17%)** |
| **Ephx1** | **0.80 ± 0.19** | **0.97 ± 0.22* (+21%)** |
| **Ephx2** | **0.85 ± 0.26** | **1.03 ± 0.40 (+21%)** |
| **Sult1e1** | **ND** | **ND** |
| **CELL PROLIFERATION/APOPTOSIS** | | |
| **Tacstd1** | **1.21 ± 0.29** | **1.20 ± 0.26 (NC)** |
| **Gadd45b** | **1.38 ± 0.42** | **1.57 ± 0.41 (+14%)** |
| **Rb1** | **1.02 ± 0.14** | **1.16 ± 0.22 (+14%)** |

NC: no change; *: p≤0.05; **: p≤0.01; ND: considered as not detected due to the variable and high Ct values, which were greater than or equal to the cut-off limit of 35 for the majority of samples (24/30 for controls and phenobarbital). In the above table the presented % changes do not always calculate exactly from the presented mean data. This is due to rounding-up differences.

## III. CONCLUSION

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.

| | |
|---|---|
| **Report:** | **KIIA 5.5.4 /33; [redacted] 2013; M-450156-01** |
| Title: | Fluopyram: Enzyme and DNA-synthesis induction in cultured human hepatocytes |
| Report No & Document No | CXR1241<br>M-450156-01 |
| **Guidelines:** | **No specific guideline** |
| **Deviations:** | **none** |
| **GLP** | **no** |

**Executive summary**


- Female human hepatocytes were cultured in the presence of the test item fluopyram, and phenobarbital (PB) or epidermal growth factor (EGF) as two positive control compounds.

- A decrease in ATP concentration commencing at 100 µM with a slight decrease 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.

# I. MATERIALS AND METHODS

## A. MATERIALS:

**1. Test Material:** Fluopyram (AE C656948)
**Description:** Beige powder
**Lot/Batch:** EDFL013235
**Purity:** 98.7 %
**CAS:** 658066-35-4
**Stability of test compound:** Stable in 0.1% (v/v), 1 µL Dimethyl sulfoxide (DMSO)/mL medium, prepared daily

**2. Vehicle and /or positive control:** Phenobarbital sodium salt (PB), positive control
**Lot/Batch:** P-5178
**Supplier:** Sigma Chemicals
**CAS:** 50-06-6
**Stability of test compound:** Stable in 0.1% (v/v), 1 µL Dimethyl sulfoxide (DMSO)/mL medium, prepared daily

**3. Vehicle and /or positive control:** Epidermal growth factor (EGF), positive control
**Lot/Batch:** E-9644
**Supplier:** Sigma Chemicals
**Stability of test compound:** Stable in 0.1% (v/v), 1 µL Dimethyl sulfoxide DMSO)/mL medium, prepared daily

**4. Test system:**
**Species**: Primary female human hepatocytes, plateable cryopreserved
**Viability:** In excess of 70% (one donor was used)
**Source:** Invitrogen, [redacted], UK

## B. STUDY DESIGN:

**1. Experimental period:** December 7 to 20, 2012.

**2. Study outline and treatment**

This study investigated the potential of fluopyram to induce the characteristic effects of CAR/PXR activation in cultured human hepatocytes. Stimulation of CYP2B activity, CYP3A activity and cell proliferation (measured as the change in replicative DNA synthesis [S-phase of the cell cycle]) were determined in primary cultures of isolated female human hepatocytes. Phenobarbital (PB) and epidermal growth factor (EGF) were included as positive control reference items for induction of CYP2B/3A activities (phenobarbital only) and cell proliferation.

**Table 5.5.4-40: Study design**

| Group # | Treatment | BrdU for S-phase | CYP Enzyme Activity | ATP |
|---|---|---|---|---|
| 1 | Control | ✓ | ✓ | ✓ |
| 2 | PB 10µM | ✓ | ✓ | ✓ |
| 3 | PB 100µM | ✓ | ✓ | ✓ |
| 4 | PB 1000µM | ✓ | ✓ | ✓ |
| 5 | Fluopyram 1µM | ✓ | ✓ | ✓ |
| 6 | Fluopyram 3µM | ✓ | ✓ | ✓ |
| 7 | Fluopyram 10µM | ✓ | ✓ | ✓ |
| 8 | Fluopyram 30µM | ✓ | ✓ | ✓ |
| 9 | Fluopyram 100µM | ✓ | ✓ | ✓ |
| 10 | Fluopyram 300µM | ✓ | ✓ | ✓ |
| 11 | EGF (+ve control BrdU) | ✓ | - | - |

**3. Statistics**

Statistical comparisons between treated hepatocytes and their control group were undertaken for all numerical data sets using a 2-tailed Student's t-test.

## C. METHODS:

**1. Hepatocyte Culture**

Primary monolayer cultures of hepatocytes were prepared in collagen coated 25 $cm^2$ flasks, 96- and 6-well plastic tissue culture plates, using Leibowitz HCL15 (Mitchell A.M. *et al* (1984) Arch. Toxicol. 55, 239-246) as the medium. In all 96-well plate cultures the outside wells were not used, but filled with sterile phosphate buffered saline.

Hepatocytes were resuscitated in Cryopreserved Hepatocyte Recovery Medium (CHRM®) then cultured in Cryopreserved Hepatocyte Plating Medium (CHPM) for approximately 6 h to allow adherence. Following a visual check for adhesion, the medium was then changed to Leibowitz HCL15 (Laboratory Method Sheet (LMS) Tic-002) medium and the hepatocytes were exposed to PB at 3 concentrations (10, 100 and 1000 µM), to fluopyram at 6 concentrations (1, 3, 10, 30, 100 and 300 µM, determined in the preliminary dose-ranging study) and to EGF (25 ng/mL). A vehicle control (0.1% v/v DMSO) was included. The media, with test or reference items, was replenished daily for a further 3 days. There were 3 replicates for each concentration in 25 $cm^2$ flasks for enzyme activity measurements, 5 replicates for each concentration in 6-well plates for replicative DNA synthesis (S-phase) analysis and 6 replicates for each concentration in 96-well plates for cell toxicity (ATP) measurements.

The test and reference items were formulated in DMSO, and administered such that the final DMSO concentration in all cultures was 0.1% (v/v), 1 µL DMSO/mL medium.

## 2. Assays

After 96 hours in culture, hepatocytes were fixed, or harvested by scraping them into SET buffer (0.25 M sucrose, 5 mM EDTA, 20 mM Tris-HCl, pH7.4), sonicating the mixture and storing it at approximately –70°C until analysis. Protein was determined by the method of Lowry *et al* (1951) J. Biol. Chem. 193, 265-275, (LMS Spec-001).

### 2.1 Adenosine 5'-Triphosphate (ATP)

Hepatocyte toxicity was assessed following 96 hours of culture as indicated by ATP depletion. Cellular ATP was determined by luminometry according to LMS FLUOR003, using an assay kit supplied by Promega (CellTitre-Glo luminescent cell viability assay).

Results are expressed as a percentage of the maximum amount of ATP released (i.e. the value of control cells).

### 2.2 Replicative DNA Synthesis (S-Phase)

The number of hepatocytes undergoing replicative DNA synthesis (S-phase) in any given cell population can be determined by the incorporation of BrdU followed by immunostaining. S-phase was determined immunocytochemically following the incorporation of BrdU into hepatocyte nuclei over the last 3 days of culture. Immunostaining was performed after fixation at 96 h. Data are expressed as a labelling index (% of total hepatocytes that have incorporated BrdU).

EGF (25 ng/mL, n=5) was included as a positive control for induction of replicative DNA synthesis.

### 2.3 Pentoxyresorufin-*O*-depentylation (PROD)

The activity of CYP2B in cultured hepatocytes was determined spectrofluorometrically by the formation of resorufin from pentoxyresorufin, as described by Burke *et al* (1985) Biochem. Pharacol. 34, 18.3337-3345, according to LMS Fluor-002.

### 2.4 Benzyloxyresorufin-*O*-debenzylation (BROD)

The activity of CYP2B/3A in cultured hepatocytes was determined spectrofluorometrically by the formation of resorufin from benzyloxyresorufin, as described by Burke *et al* (1985), according to LMS Fluor-002.

### 2.5 Benzyloxyquinoline-*O*-debenzylation (BQ)

The activity of CYP3A in cultured hepatocytes was determined spectrofluorometrically by the formation of 7-hydroxyquinoline from benzyloxyquinoline, as described by GENTEST HTS technical bulletin, according to LMS Fluor-005.

## II. RESULTS AND DISCUSSION

### 1. Adenosine 5'-Triphosphate (ATP)

Fluopyram (1 - 30 µM) had little, or no, effect on cellular ATP concentrations after 96 hours. However, at a concentration of 100 µM a slight decrease in in ATP concentration was observed, whilst at 300 µM a marked decrease in ATP concentration was observed, indicating the start of cytotoxicity at 100 µM, which was clearly evident at 300 µM.

**Table 5.5.4-41: Adenosine 5'-Triphosphate**

| Treatment | ATP (% Control) |
|---|---|
| Vehicle Control | 100.0 ± 8.7[a] |
| PB 10 µM | 110.0 ± 5.2* |
| PB 100 µM | 103.4 ± 7.1 |
| PB 1000 µM | 110.8 ± 9.5 |
| Fluopyram 1 µM | 108.9 ± 1.6* |
| Fluopyram 3 µM | 113.0 ± 6.3* |
| Fluopyram 10 µM | 111.5 ± 11.5 |
| Fluopyram 30 µM | 126.3 ± 8.6*** |
| Fluopyram 100 µM | 91.9 ± 6.9 |
| Fluopyram 300 µM | 29.1 ± 1.4*** |

[a] Values are mean % control ± SD. n = 6 per group. A Student's t-test (2-sided) was performed on the results; *statistically different from control p<0.05; ** p<0.01; ***p<0.001.

**Figure 5.5.4-1: Adenosine 5'-Triphosphate**

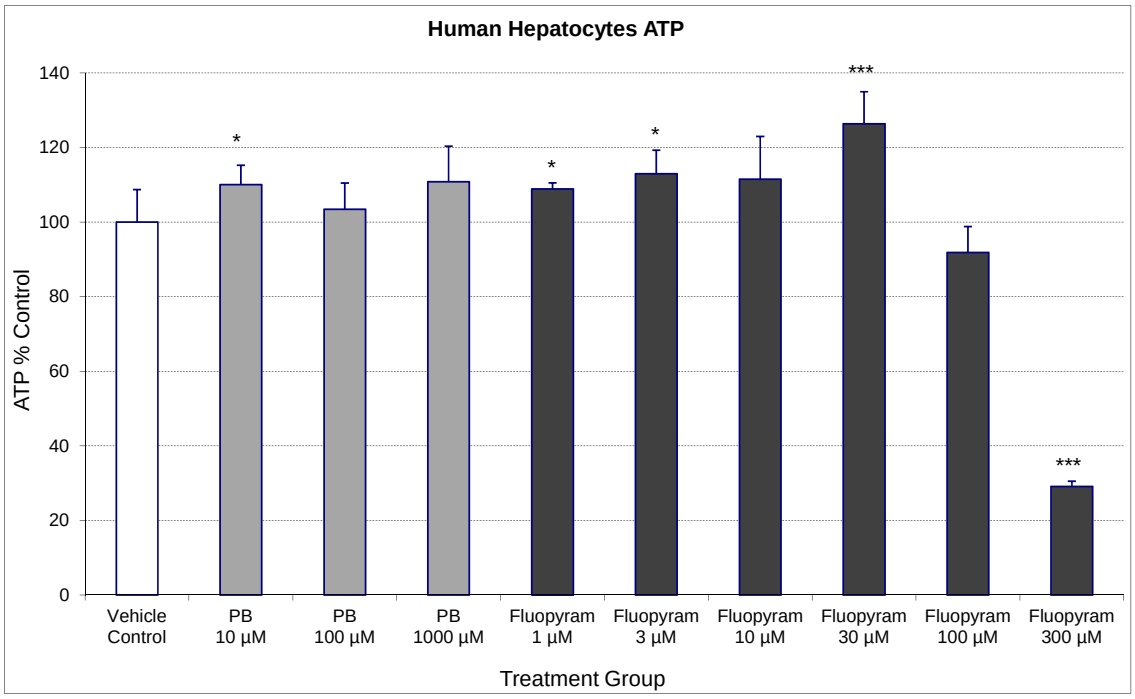
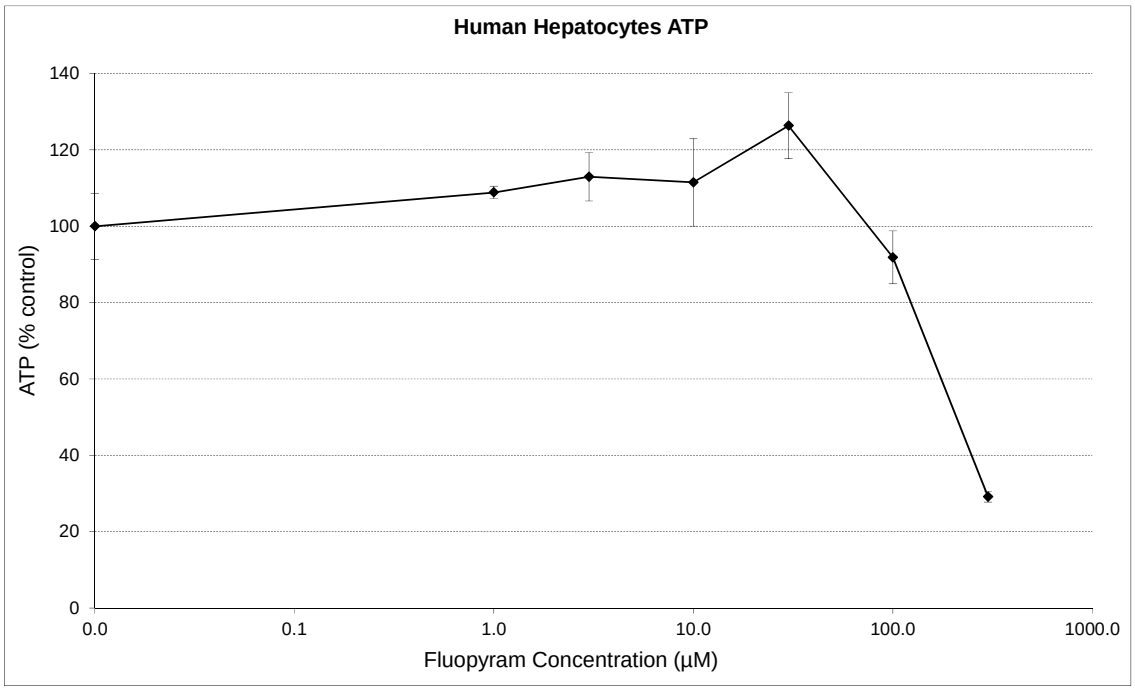


**Figure 5.5.4-2:** Adenosine 5'-Triphosphate, Fluopyram Dose Response




## 2. Replicative DNA Synthesis (S-Phase)

Treatment with 25ng/mL EGF resulted in a statistically significant increase in replicative DNA synthesis to 15-fold control values. This indicates that the hepatocytes could proliferate following exposure to proliferative stimuli, and therefore demonstrating their suitability for use in investigations involving assessing induction of proliferation.

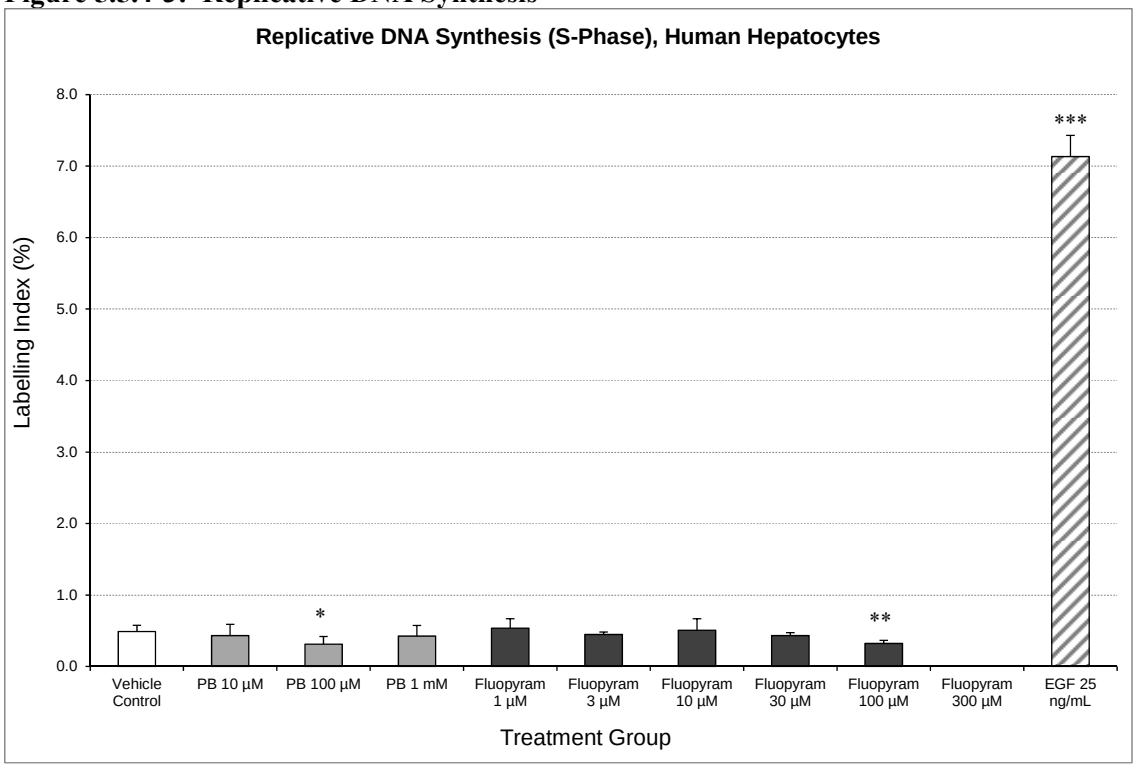
No biologically significant changes in replicative DNA synthesis were observed following treatment with either PB (10 – 1000 µM) or fluopyram (1 - 30 µM). However, at a concentration of 100 µM fluopyram, there was a decrease in hepatocyte confluency, with fewer adherent hepatocytes present, possibly indicating the beginning of dose-dependent toxicity. Analysis of the cells exposed to 300 µM fluopyram was precluded due to extensive cytotoxicity.

**Table 5.5.4-42: Replicative DNA Synthesis**

| Treatment | Labelling Index (%) |
|---|---|
| Vehicle Control | 0.49 ± 0.09 (100.0 ± 18.5)[a] |
| PB 10 µM | 0.43 ± 0.16 (88.4 ± 32.4) |
| PB 100 µM | 0.31 ± 0.11* (63.8 ± 22.2) |
| PB 1000 µM | 0.43 ± 0.15 (87.4 ± 30.3) |
| Fluopyram 1 µM | 0.54 ± 0.13 (109.9 ± 27.2) |
| Fluopyram 3 µM | 0.45 ± 0.03 (91.7 ± 7.1) |
| Fluopyram 10 µM | 0.51 ± 0.16 (103.7 ± 33.2) |
| Fluopyram 30 µM | 0.43 ± 0.04 (88.7 ± 7.9) |
| Fluopyram 100 µM | 0.32 ± 0.04** (66.1 ± 9.1)[b] |
| Fluopyram 300 µM | [c] |
| EGF 25ng/mL | 7.13 ± 0.30*** (1461.4 ± 60.7) |

[a] Values are Mean ± SD. Values in parenthesis are mean % control ± SD n = 5 per group. [b] Decrease in hepatocyte confluency, fewer adherent hepatocytes present. [c]Analysis unable to be performed due to excessive cytotoxicity. A Student's t-test (2-sided) was performed on the results; *statistically different from control p<0.05; ** p<0.01; ***p<0.001.

**Figure 5.5.4-3: Replicative DNA Synthesis**



### 3. Pentoxyresorufin-*O*-Depentylation (PROD)

Culturing primary human hepatocytes for 96 hours in the presence of phenobarbital resulted in increases in PROD activity to a maximum 3.1-fold at 1000 µM. This is consistent with historical data.

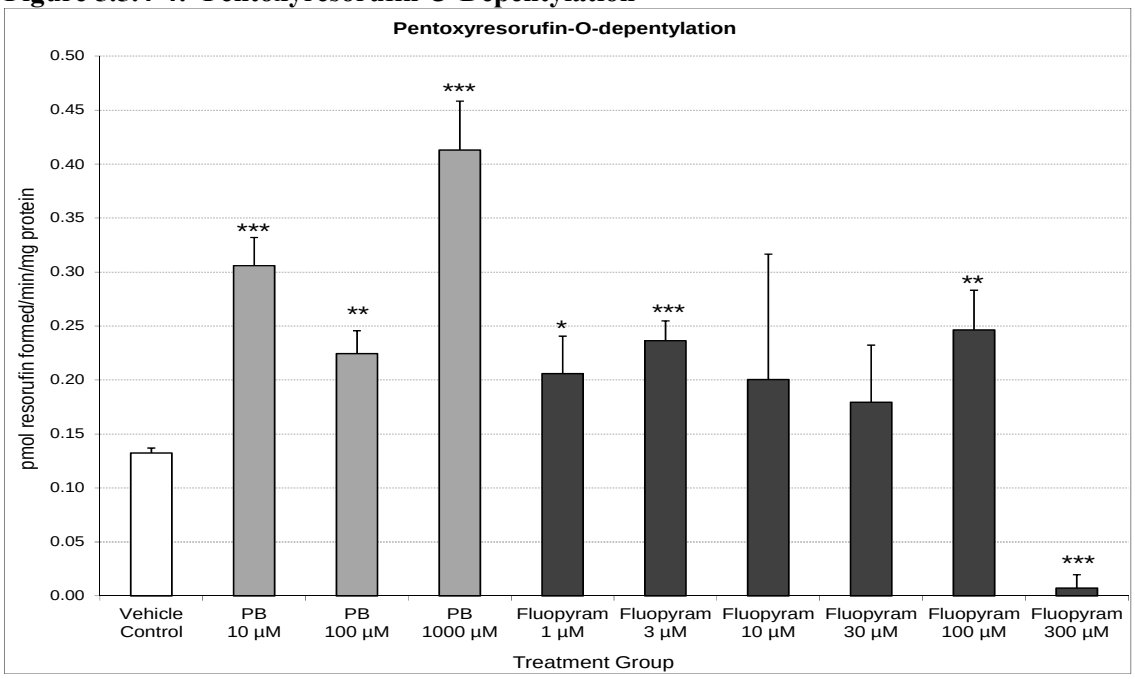
Addition of fluopyram to the hepatocytes resulted in increases in PROD activity to a maximum 1.9-fold of control value at 100 µM. Treatment with 300 µM fluopyram reduced PROD activity to 5% of the control value, reflecting the cytotoxicity shown by the ATP and S-phase results (Table 4 and Figure 4). Clear dose-dependent induction by fluopyram was not seen, suggesting that maximal effects were seen at the low concentrations of fluopyram.

**Table 5.5.4-43: Pentoxyresorufin-*O*-Depentylation**

| Treatment | PROD (pmol resorufin formed/min/mg protein) |
|---|---|
| Vehicle Control | 0.132 ± 0.005 (100.0 ± 3.5)[a] |
| PB 10 µM | 0.306 ± 0.026*** (231.1 ± 19.8) |
| PB 100 µM | 0.224 ± 0.021** (169.6 ± 16.0) |
| PB 1000 µM | 0.413 ± 0.045*** (312.0 ± 34.2) |
| Fluopyram 1 µM | 0.206 ± 0.035* (155.6 ± 26.2) |
| Fluopyram 3 µM | 0.236 ± 0.018*** (178.6 ± 13.9) |
| Fluopyram 10 µM | 0.200 ± 0.116 (151.4 ± 87.7) |
| Fluopyram 30 µM | 0.179 ± 0.053 (135.5 ± 40.0) |
| Fluopyram 100 µM | 0.246 ± 0.037** (186.1 ± 27.8) |
| Fluopyram 300 µM | 0.007 ± 0.012*** (5.4 ± 9.4) |

[a] Values are Mean ± SD. Values in parenthesis are mean % control ± SD. n = 3 per group. A Student's t-test (2-sided) was performed on the results; *statistically different from control $p<0.05$; ** $p<0.01$; ***$p<0.001$.

**Figure 5.5.4-4: Pentoxyresorufin-*O*-Depentylation**




## 4. Benzyloxyresorufin-*O*-Debenzylation (BROD)

Culturing primary human hepatocytes, for 96 hours, with phenobarbital resulted in increases in BROD activity up to a maximum 4-fold increase at 1000 µM. This is consistent with historical data.

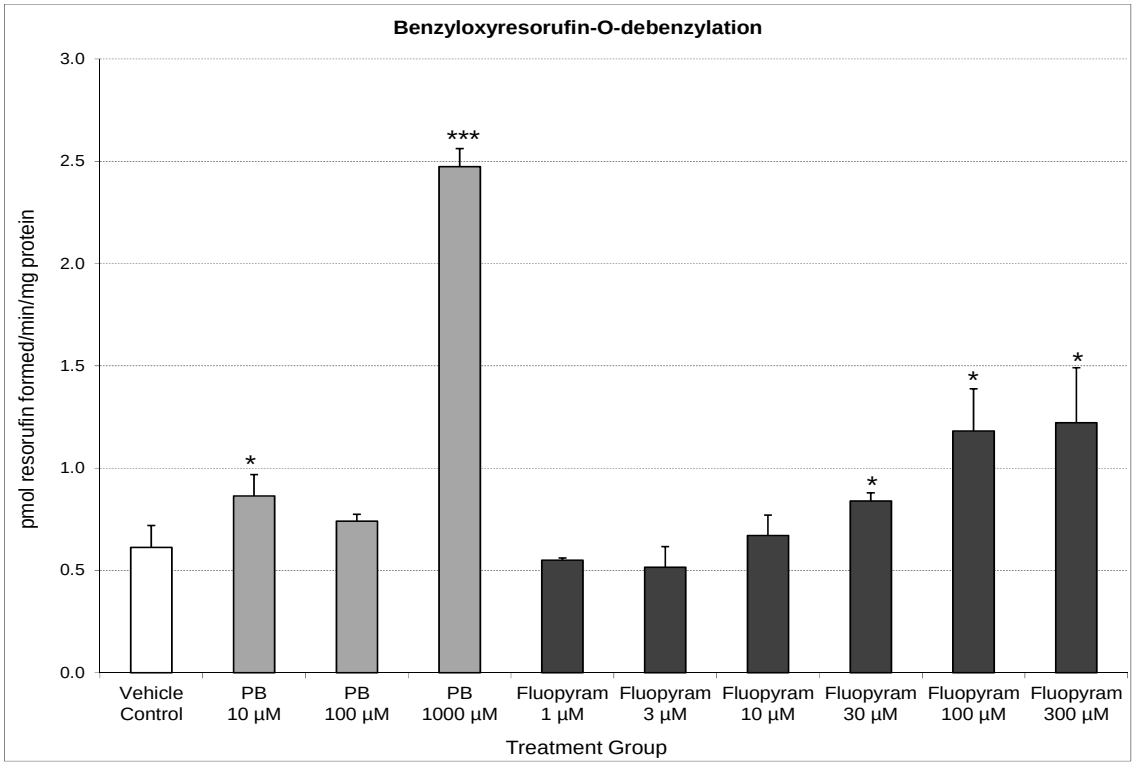
Culturing hepatocytes with fluopyram resulted in a dose-dependent increase in BROD activity, up to a maximum increase of 2-fold at 300 µM. This level of induction could be affected by the cytotoxicity shown by the ATP and S-phase and PROD results.

**Table 5.5.4-44: Benzyloxyresorufin-*O*-Debenzylation**

| Treatment | BROD (pmol resorufin formed/min/mg protein) |
|---|---|
| Vehicle Control | 0.612 ± 0.107 (100.0 ± 17.5)[a] |
| PB 10 µM | 0.865 ± 0.105* (141.2 ± 17.1) |
| PB 100 µM | 0.741 ± 0.033 (121.0 ± 5.5) |
| PB 1000 µM | 2.473 ± 0.089*** (403.8 ± 14.5) |
| Fluopyram 1 µM | 0.550 ± 0.011 (89.9 ± 1.8) |
| Fluopyram 3 µM | 0.515 ± 0.102 (84.1 ± 16.6) |
| Fluopyram 10 µM | 0.671 ± 0.100 ( 109.5± )16.4 |
| Fluopyram 30 µM | 0.840 ± 0.040* (137.1 ± 6.5) |
| Fluopyram 100 µM | 1.182 ± 0.206* (193.0 ± 33.6) |
| Fluopyram 300 µM | 1.222 ± 0.269* (199.5 ± 43.9) |

[a] Values are Mean ± SD. Values in parenthesis are mean % control ± SD. n = 3 per group. A Student's t-test (2-sided) was performed on the results; *statistically different from control p<0.05; ** p<0.01; ***p<0.001.

**Figure 5.5.4-5: Benzyloxyresorufin-*O*-Debenzylation**




## 5. Benzyloxyquinoline-*O*-Debenzylation (BQ)

Culturing primary human hepatocytes, for 96 hours, with phenobarbital resulted in a dose-dependent increase in BQ activity of up to 5.2-fold at 1000 µM. This is consistent with historical data.

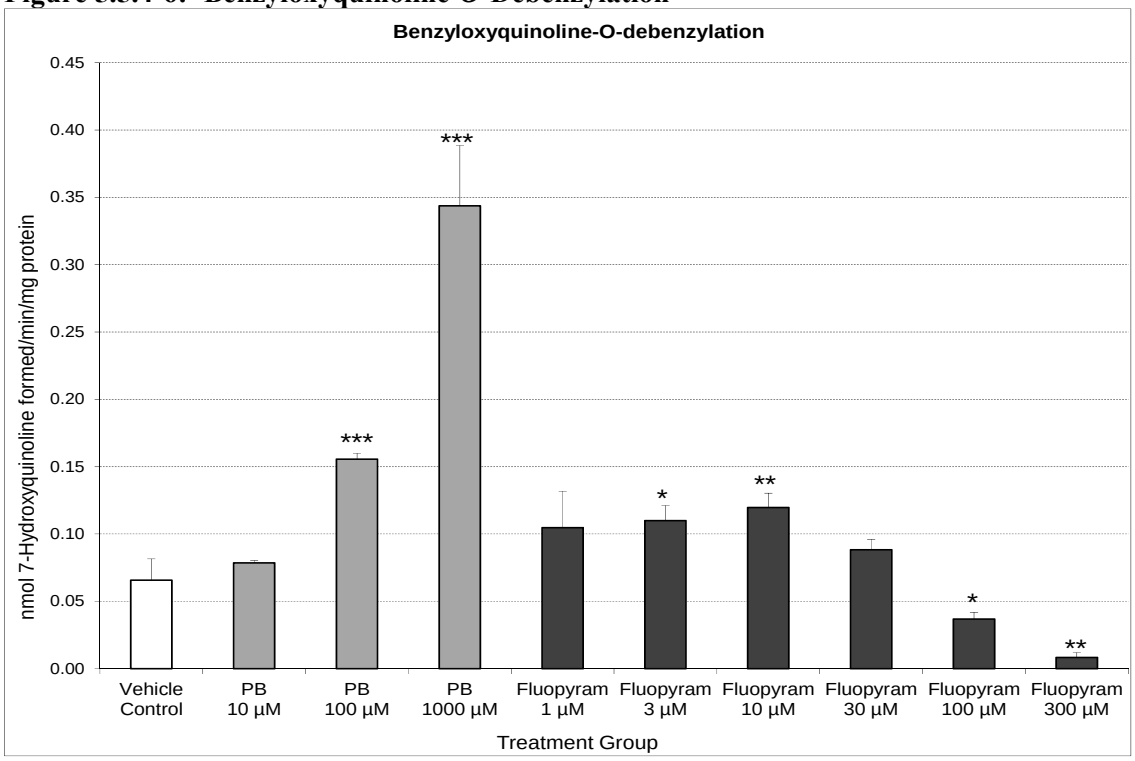
Exposure of hepatocytes to fluopyram resulted in an increase in benzyloxyquinoline-*O*-debenzylation (BQ) activity up to a maximum of 1.8–fold at 10 µM. This indicates that fluopyram is an inducer of CYP3A at low concentrations. Higher concentrations led to decreases in activity, with cytotoxicity commencing at 100 µM and clearly evident at 300 µM.

**Table 5.5.4-45: Benzyloxyquinoline-*O*-Debenzylation**

| Treatment | BQ (nmol 7-Hydroxyquinoline formed/min/mg protein) |
|---|---|
| Vehicle Control | 0.066 ± 0.016 (100.0 ± 24.2)[a] |
| PB 10 µM | 0.079 ± 0.002 (119.6 ± 2.5) |
| PB 100 µM | 0.156 ± 0.004*** (236.8 ± 6.5) |
| PB 1000 µM | 0.344 ± 0.045*** (523.3 ± 67.9) |
| Fluopyram 1 µM | 0.105 ± 0.027 (159.4 ± 41.1) |
| Fluopyram 3 µM | 0.110 ± 0.011* (167.5 ± 16.9) |
| Fluopyram 10 µM | 0.120 ± 0.011** (182.2 ± 16.1) |
| Fluopyram 30 µM | 0.088 ± 0.008 (134.4 ± 11.6) |
| Fluopyram 100 µM | 0.037 ± 0.005* (56.2 ± 7.2) |
| Fluopyram 300 µM | 0.008 ± 0.003** (12.7 ± 5.3) |

[a] Values are Mean ± SD. Values in parenthesis are mean % control ± SD n = 3 per group. A Student's t-test (2-sided) was performed on the results; *statistically different from control p<0.05; ** p<0.01; ***p<0.001.

**Figure 5.5.4-6: Benzyloxyquinoline-*O*-Debenzylation**



## III. CONCLUSION

Fluopyram and phenobarbital appeared to exhibit similar properties in human hepatocytes, inducing cytochromes P450 *via* the Pregnane X Receptor (PXR) and the Constitutive Androstane Receptor (CAR). The increases in PROD activity seen after exposure to even low concentrations of fluopyram indicate that fluopyram is an inducer of CYP2B. The induction of BROD (at higher concentrations) and BQ (at lower concentrations) activity suggests that fluopyram is also a CYP3A inducer.

Human CAR/PXR did not mediate compound-stimulated DNA synthesis in human hepatocytes. Neither phenobarbital nor fluopyram stimulated replicative DNA synthesis (S-phase) in the hepatocytes, whilst the positive control EGF elicited a 15-fold increase in S-phase.

In conclusion, these data suggest that fluopyram is a weak activator of CAR and PXR, with no compound-stimulated DNA synthesis in human hepatocytes.

| | |
|---|---|
| **Report:** | **KIIA 5.5.4 /32; [redacted]; 2013; M-450157-01** |
| Title: | Fluopyram: Enzyme and DNA-synthesis induction in cultured rat hepatocytes |
| Report No & | CXR1242 |
| Document No | M-450157-01 |
| **Guidelines:** | **No specific guideline** |
| **Deviations:** | **none** |
| **GLP** | **no** |

**Executive summary**


- 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.

# I. MATERIALS AND METHODS

## A. MATERIALS:

**1. Test Material:** Fluopyram (AE C656948)
**Description:** Beige powder
**Lot/Batch:** EDFL013235
**Purity:** 98.7 %
**CAS:** 658066-35-4
**Stability of test compound:** Stable in 0.1% (v/v), 1 µL Dimethyl sulfoxide (DMSO)/mL medium, over period of usage, prepared daily

**2. Vehicle and /or positive control:** Phenobarbital sodium salt (PB), positive control
**Lot/Batch:** P-5178
**Supplier:** Sigma Chemicals
**CAS:** 50-06-6
**Stability of test compound:** Stable in 0.1% (v/v) 1 µL DMSO/mL medium, over period of usage, prepared daily

**3. Vehicle and /or positive control:** Epidermal growth factor (EGF), positive control
**Lot/Batch:** E-9644
**Supplier:** Sigma Chemicals
**Stability of test compound:** Stable in 0.1% (v/v) 1 µL DMSO/mL medium, over period of usage, prepared daily

**4. Test animals:**
**Species**: Rat – Female only
**Strain:** Han Wistar (out-bred HsdHan™: WIST)
**Age:** 8 weeks approximately at start of treatment
**Weight at start of dosing:** 150 to 200 g at arrival
**Acclimation period:** at least 5 days
**Diet:** RM1pelleted diet ([redacted], [redacted], UK), *ad libitum*
**Water:** Tap water, *ad libitum*
**Housing:** Animals were housed in groups on saw-dust in solid-bottom, polypropylene cages.
**Environmental conditions –**
**Temperature:** 19 – 23°C
**Humidity:** 40 – 70 %
**Air changes:** Approximately 14-15 changes per hour
**Photoperiod:** Alternating 12-hour light and dark cycles

**Test system:** Primary monolayer cultures of hepatocytes
**Viability:** In excess of 80% (hepatocytes from two independent perfusions were pooled)

**B. STUDY DESIGN:**

**1. Experimental period:** November 22 to December 20, 2012.

**2. Study outline and treatment**

This study investigated the potential of fluopyram to induce the characteristic effects of CAR/PXR activation in cultured rat hepatocytes. Stimulation of CYP2B activity, CYP3A activity and cell proliferation (measured as the change in replicative DNA synthesis [S-phase of the cell cycle]) were determined in primary cultures of isolated female rat hepatocytes. Phenobarbital (PB) and epidermal growth factor (EGF) were included as positive control reference items for induction of CYP2B/3A activities (phenobarbital only) and cell proliferation.

**Table 5.5.4-46: Study design**

| Group # | Treatment | BrdU for S-phase | CYP Enzyme Activity | ATP |
|---|---|---|---|---|
| 1 | Control | ✓ | ✓ | ✓ |
| 2 | PB 10µM | ✓ | ✓ | ✓ |
| 3 | PB 100µM | ✓ | ✓ | ✓ |
| 4 | PB 1000µM | ✓ | ✓ | ✓ |
| 5 | Fluopyram 1µM | ✓ | ✓ | ✓ |
| 6 | Fluopyram 3µM | ✓ | ✓ | ✓ |
| 7 | Fluopyram 10µM | ✓ | ✓ | ✓ |
| 8 | Fluopyram 30µM | ✓ | ✓ | ✓ |
| 9 | Fluopyram 100µM | ✓ | ✓ | ✓ |
| 10 | Fluopyram 300µM | ✓ | ✓ | ✓ |
| 11 | EGF (+ve control BrdU) | ✓ | - | - |

**3. Statistics**

Statistical comparisons between treated hepatocytes and their control group were undertaken for all numerical data sets using a 2-tailed Student's t-test.

**A. METHODS:**

**1. Hepatocyte Isolation**

Rats were terminally anaesthetised, using euthatal, and hepatocytes isolated by *in situ* perfusion according to Mitchell A.M. *et al* (1984) Arch. Toxicol. 55: 239-246. Viabilities of the hepatocyte preparations, determined by trypan blue exclusion, must be in excess of 80%. Hepatocytes from two independent perfusions were pooled.

**2. Hepatocyte Culture**

Primary monolayer cultures of hepatocytes were prepared in 25 $cm^2$ flasks, 96- and 6-well plastic tissue culture plates, using Leibowitz HCL15 (Mitchell A.M. *et al* (1984) Arch. Toxicol. 55, 239-

246) as the medium. In all 96-well plate cultures the outside wells were not used, but filled with sterile phosphate buffered saline.

Hepatocytes were cultured in Leibowitz CL15 (Laboratory Method Sheet (LMS) TIC001) medium for approximately 4h to allow adhesion. Following a visual check for adhesion, the medium was changed and the hepatocytes exposed to phenobarbital sodium salt at 3 concentrations (10, 100 and 1000 µM), fluopyram at 6 concentrations (1, 3, 10, 30, 100 and 300 µM, determined in the preliminary dose-ranging study) and to EGF (25 ng/mL). A vehicle control (0.1% v/v DMSO) was included. The media, with test items, was replenished daily for a further 3 days. For each test and reference item, there were 3 replicates for each concentration in 25 cm2 flasks for enzyme activity measurements, 5 replicates for each concentration in 6-well plates for replicative DNA synthesis (S-phase) analysis and 6 replicates for each concentration in 96-well plates for cell toxicity (ATP) measurements. The test and reference items were formulated in DMSO, and administered such that the final DMSO concentration in all cultures was 0.1%, 1 µL DMSO/mL medium.

**3. Assays**

After 96 hours in culture hepatocytes were fixed, or harvested by scraping them into SET buffer (0.25 M sucrose, 5 mM EDTA, 20 mM Tris-HCl, pH 7.4), sonicating the mixture and storing it at –70oC until analysis. Protein was determined by the method of Lowry *et al* (1951) J. Biol. Chem. **193**, 265-275 (MET-SPEC001).

**4. Adenosine 5'-Triphosphate (ATP)**

Hepatocyte toxicity was assessed following 96 hours of culture as indicated by ATP depletion. The ATP released from viable somatic cells was determined by luminometry according to LMS FLUOR003, using an assay kit supplied by Promega (CellTitre-Glo luminescent cell viability assay).

Results are expressed as a percentage of the maximum amount of ATP released (i.e. the value of control cells).

**5. Replicative DNA Synthesis (S-Phase)**

The number of hepatocytes undergoing replicative DNA synthesis (S-phase) in any given cell population can be determined by the incorporation of BrdU followed by immunostaining. S-phase will be determined immunocytochemically following the incorporation of BrdU into hepatocyte nuclei over the last 3 days of culture. Immunostaining was performed after fixation at 96 h. Data will be expressed as a labelling index (% of total hepatocytes that have incorporated BrdU).

EGF (25 ng/mL, n=5) was included as a positive control for induction of replicative DNA synthesis.

**6. Pentoxyresorufin-*O*-depentylation (PROD)**

The activity of CYP2B in cultured hepatocytes was determined spectrofluorometrically by the formation of resorufin from pentoxyresorufin, as described by Burke *et al* (1985) Biochem. Pharmacol. **34**, 18. 3337-3345, according to LMS Fluor-002.

### 7. Benzyloxyresorufin-*O*-debenzylation (BROD)

The activity of CYP2B/3A in cultured hepatocytes was determined spectrofluorometrically by the formation of resorufin from benzyloxyresorufin, as described by Burke *et al* (1985), according to LMS Fluor-002.

### 8. Benzyloxyquinoline-*O*-debenzylation (BQ)

The activity of CYP3A in cultured hepatocytes was determined spectrofluorometrically by the formation of 7-hydroxyquinoline from benzyloxyquinoline, as described by GENTEST HTS technical bulletin, according to LMS Fluor-005.

## II. RESULTS AND DISCUSSION

### A. Adenosine 5'-Triphosphate (ATP)

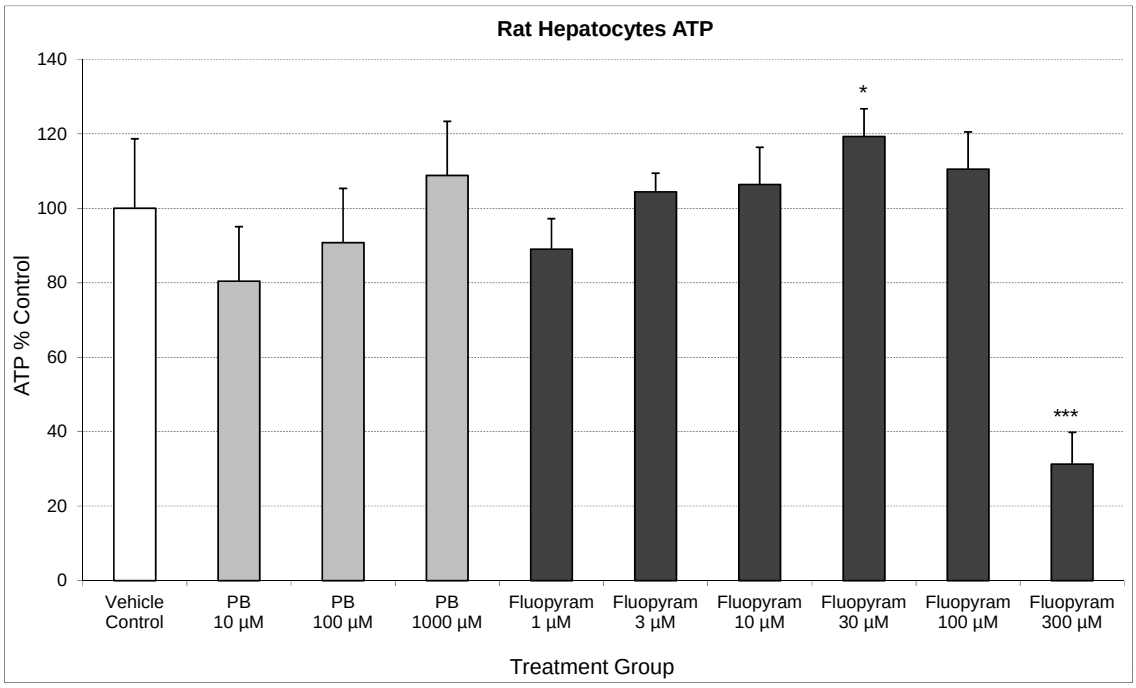
Fluopyram (1 - 100 µM) had little, or no, effect on cellular ATP concentrations after 96 hours. However, at a concentration of 300 µM a marked decrease in ATP concentration was observed, demonstrating cytotoxicity.

**Table 5.5.4-47: Adenosine 5'-Triphosphate**

| Treatment | ATP (% Control) |
|---|---|
| Vehicle Control | 100.0 ± 18.7[a] |
| PB 10 µM | 80.5 ± 14.6 |
| PB 100 µM | 90.8 ± 14.6 |
| PB 1000 µM | 108.9 ± 14.5 |
| Fluopyram 1 µM | 89.0 ± 8.2 |
| Fluopyram 3 µM | 104.4 ± 5.0 |
| Fluopyram 10 µM | 106.4 ± 10.0 |
| Fluopyram 30 µM | 119.3 ± 7.4* |
| Fluopyram 100 µM | 110.5 ± 10.0 |
| Fluopyram 300 µM | 31.3 ± 8.6*** |

[a] Values are mean % control ± SD. n = 6 per group. A Student's t-test (2-sided) was performed on the results; *statistically different from control p<0.05; ** p<0.01; ***p<0.001.

**Figure 5.5.4-7: Adenosine 5'-Triphosphate**




10.2

## B. Replicative DNA Synthesis (S-Phase)

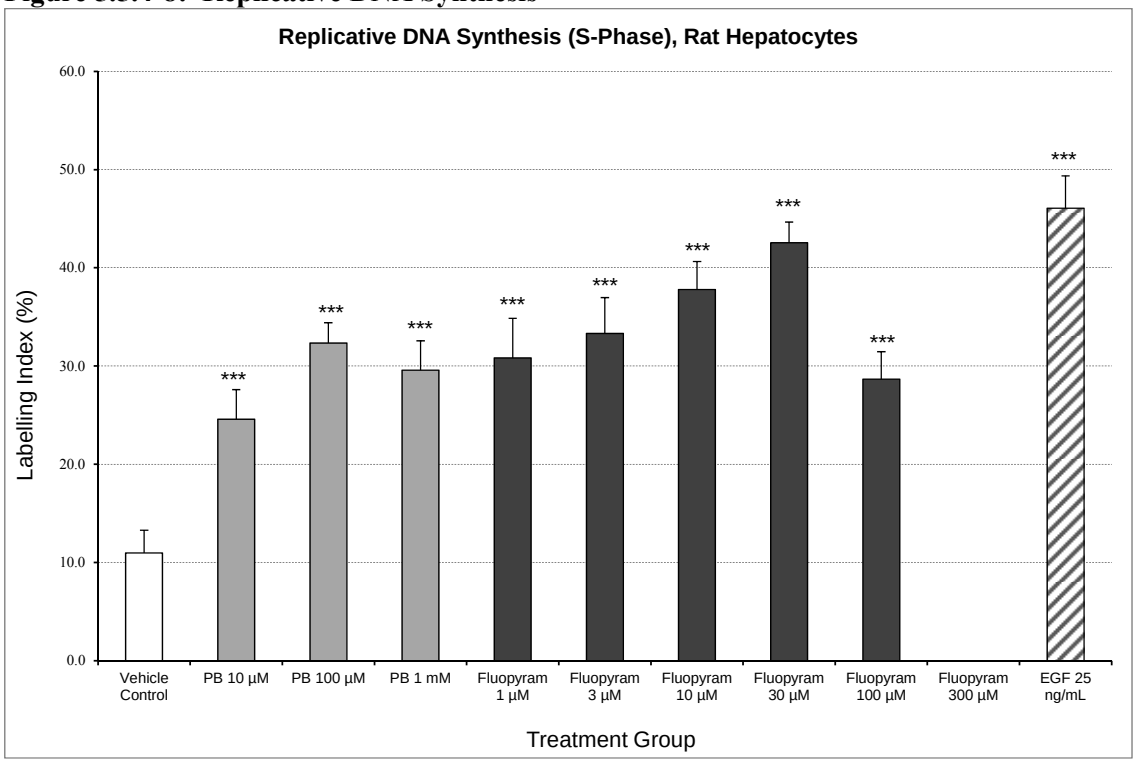
Culturing primary rat hepatocytes, for 96 hours with phenobarbital resulted in a dose- dependent increase in replicative DNA synthesis up to a maximum of 2.9-fold. Similarly, a dose-dependent increase of S-phase was observed following treatment with fluopyram, with maximal stimulation at 30 µM fluopyram (3.9-fold). Exposure of hepatocytes to higher concentrations resulted in less stimulation of S-phase. The second positive control, epidermal growth factor (EGF) increased replicative DNA synthesis by 4.2-fold, which is within the historical data range.

**Table 5.5.4-48: Replicative DNA Synthesis**

| Treatment | Labelling Index (%) |
|---|---|
| Vehicle Control | 10.97 ± 2.32 (100.0 ± 21.1)[a] |
| PB 10 µM | 24.59 ± 3.00*** (224.3 ± 27.4) |
| PB 100 µM | 32.34 ± 2.07*** (294.9 ± 18.8) |
| PB 1000 µM | 29.57 ± 2.99*** (269.7 ± 27.3) |
| Fluopyram 1 µM | 30.82 ± 4.04*** (281.0 ± 36.8) |
| Fluopyram 3 µM | 33.31 ± 3.66*** (303.8 ± 33.4) |
| Fluopyram 10 µM | 37.79 ± 2.386*** (344.6 ± 26.0) |
| Fluopyram 30 µM | 42.56 ± 2.09*** (388.1 ± 19.1) |
| Fluopyram 100 µM | 28.67 ± 2.80*** (261.4 ± 25.5) |
| Fluopyram 300 µM | [b] |
| EGF 25ng/mL | 46.07 ± 3.29*** (420.1 ± 30.0) |

[a] Values are Mean ± SD. Values in parenthesis are mean % control ± SD n = 5 per group. [b] Analysis unable to be performed due to excessive cytotoxicity. A Student's t-test (2-sided) was performed on the results; *statistically different from control p<0.05; ** p<0.01; ***p<0.001.

**Figure 5.5.4-8: Replicative DNA Synthesis**




## C. Pentoxyresorufin-*O*-Depentylation (PROD)

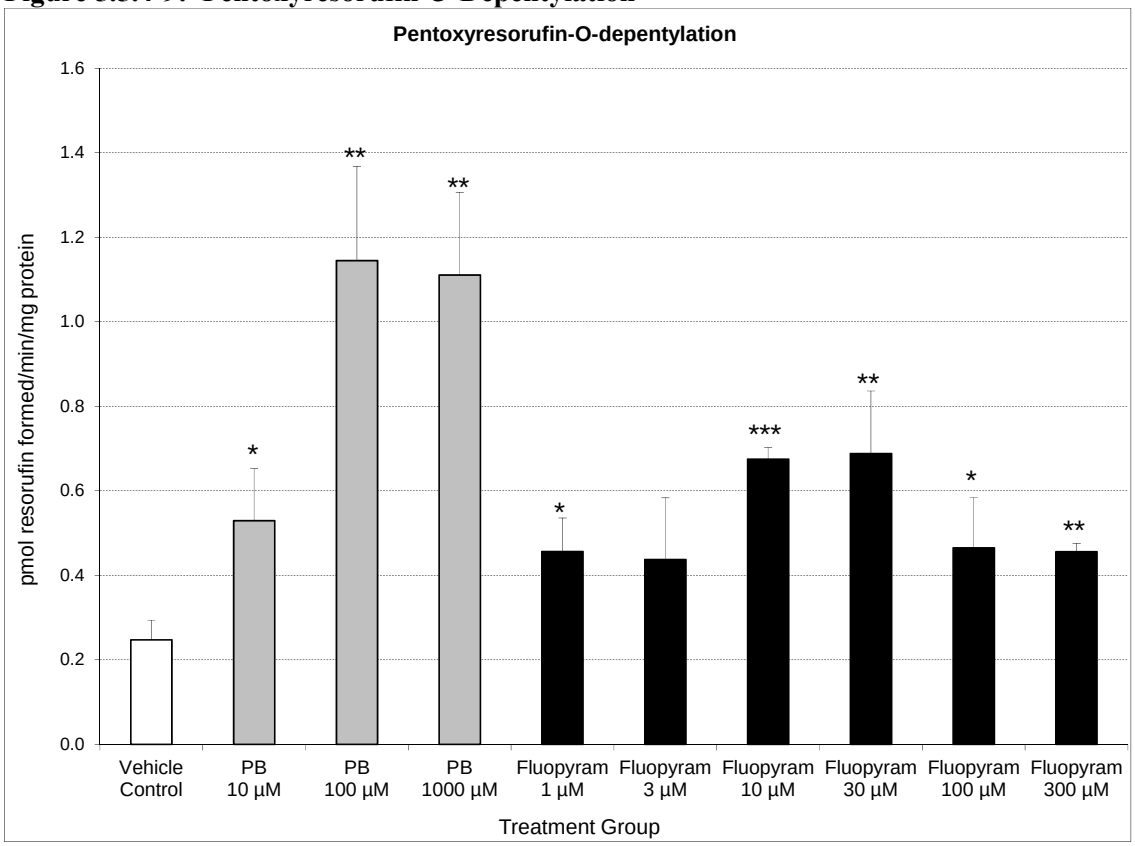
Culturing primary rat hepatocytes for 96 hours in the presence of phenobarbital resulted in a dose-dependent increase in PROD activity to a maximum 4.6-fold at 100 µM. This is consistent with historical data. Addition of fluopyram to the hepatocytes resulted in a dose-dependent increase in PROD activity to a maximum 2.8-fold of control value at 30 µM. The degree of induction decreased as the concentration of fluopyram was increased to 100 and 300 µM .

**Table 5.5.4-49: Pentoxyresorufin-*O*-Depentylation**

| Treatment | PROD (pmol resorufin formed/min/mg protein) |
|---|---|
| Vehicle Control | 0.247 ± 0.046 (100.0 ± 18.7)[a] |
| PB 10 µM | 0.529 ± 0.123* (213.9 ± 49.7) |
| PB 100 µM | 1.145 ± 0.222** (462.9 ± 89.9) |
| PB 1000 µM | 1.110 ± 0.196** (448.9 ± 79.1) |
| Fluopyram 1 µM | 0.456 ± 0.079* (184.5 ± 32.0) |
| Fluopyram 3 µM | 0.437 ± 0.146 (176.7 ± 59.1) |
| Fluopyram 10 µM | 0.675 ± 0.027*** (272.9 ± 11.0) |
| Fluopyram 30 µM | 0.688 ± 0.148** (278.2 ± 59.9) |
| Fluopyram 100 µM | 0.465 ± 0.119* (187.9 ± 48.2) |
| Fluopyram 300 µM | 0.456 ± 0.019** (184.4 ± 7.8) |

[a] Values are Mean ± SD. Values in parenthesis are mean % control ± SD. n = 3 per group. A Student's t-test (2-sided) was performed on the results; *statistically different from control p<0.05; ** p<0.01; ***p<0.001.

**Figure 5.5.4-9: Pentoxyresorufin-*O*-Depentylation**




## D. Benzyloxyresorufin-*O*-Debenzylation (BROD)

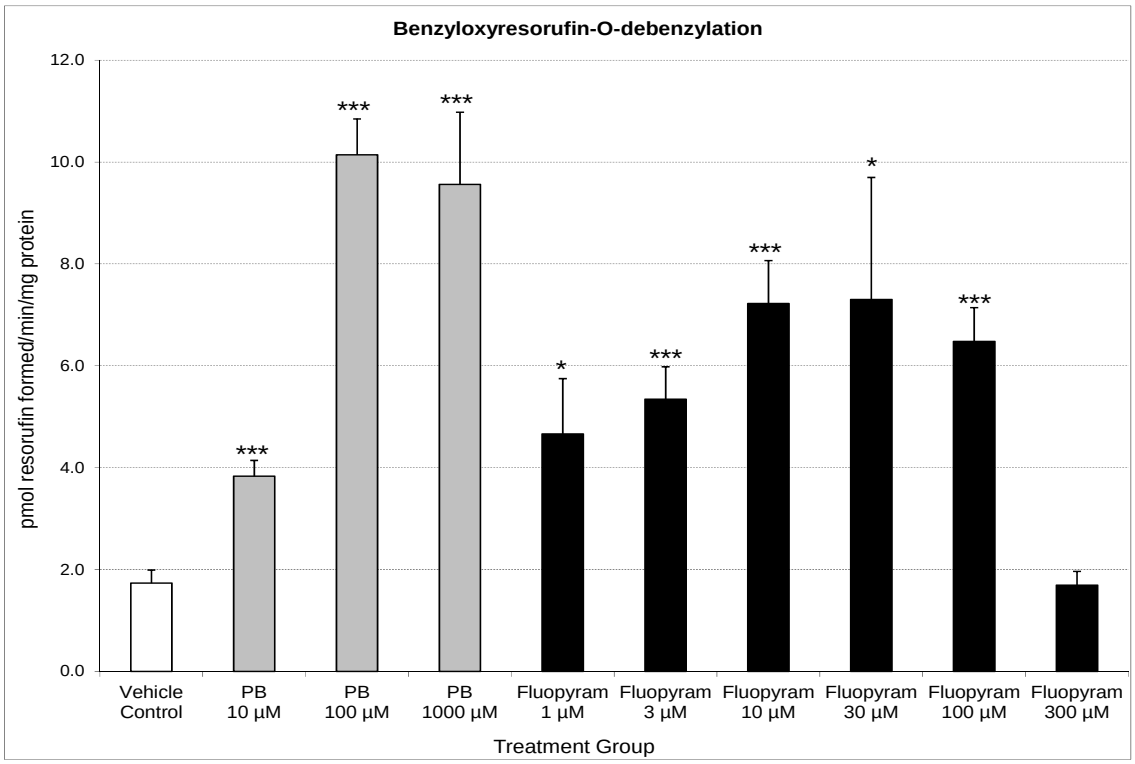
Culturing primary rat hepatocytes, for 96 hours, with phenobarbital resulted in a dose-dependent increase in BROD activity up to a maximum 5.8-fold increase at 100 µM. This is consistent with historical data. Culturing hepatocytes with fluopyram resulted in a dose-dependent increase in BROD activity, up to a maximum increase of 4.2-fold at 30 µM.

**Table 5.5.4-50: Benzyloxyresorufin-*O*-Debenzylation**

| Treatment | BROD (pmol resorufin formed/min/mg protein) |
|---|---|
| Vehicle Control | 1.734 ± 0.255 (100.0 ± 14.7)[a] |
| PB 10 µM | 3.835 ± 0.309*** (221.1 ± 17.8) |
| PB 100 µM | 10.142 ± 0.704*** (584.8 ± 40.6) |
| PB 1000 µM | 9.560 ± 1.419*** (551.3 ± 81.8) |
| Fluopyram 1 µM | 4.661 ± 1.087* (268.7 ± 62.7) |
| Fluopyram 3 µM | 5.343 ± 0.639*** (308.1 ± 36.8) |
| Fluopyram 10 µM | 7.223 ± 0.844*** (416.5 ± 48.7) |
| Fluopyram 30 µM | 7.299 ± 2.398* (420.9 ± 138.3) |
| Fluopyram 100 µM | 6.480 ± 0.662*** (373.7 ± 38.1) |
| Fluopyram 300 µM | 1.693 ± 0.217 (97.7 ± 15.7) |

[a] Values are Mean ± SD. Values in parenthesis are mean % control ± SD. n = 3 per group. A Student's t-test (2-sided) was performed on the results; *statistically different from control p<0.05; ** p<0.01; ***p<0.001.

**Figure 5.5.4-10: Benzyloxyresorufin-*O*-Debenzylation**




## E. Benzyloxyquinoline-*O*-Debenzylation (BQ)

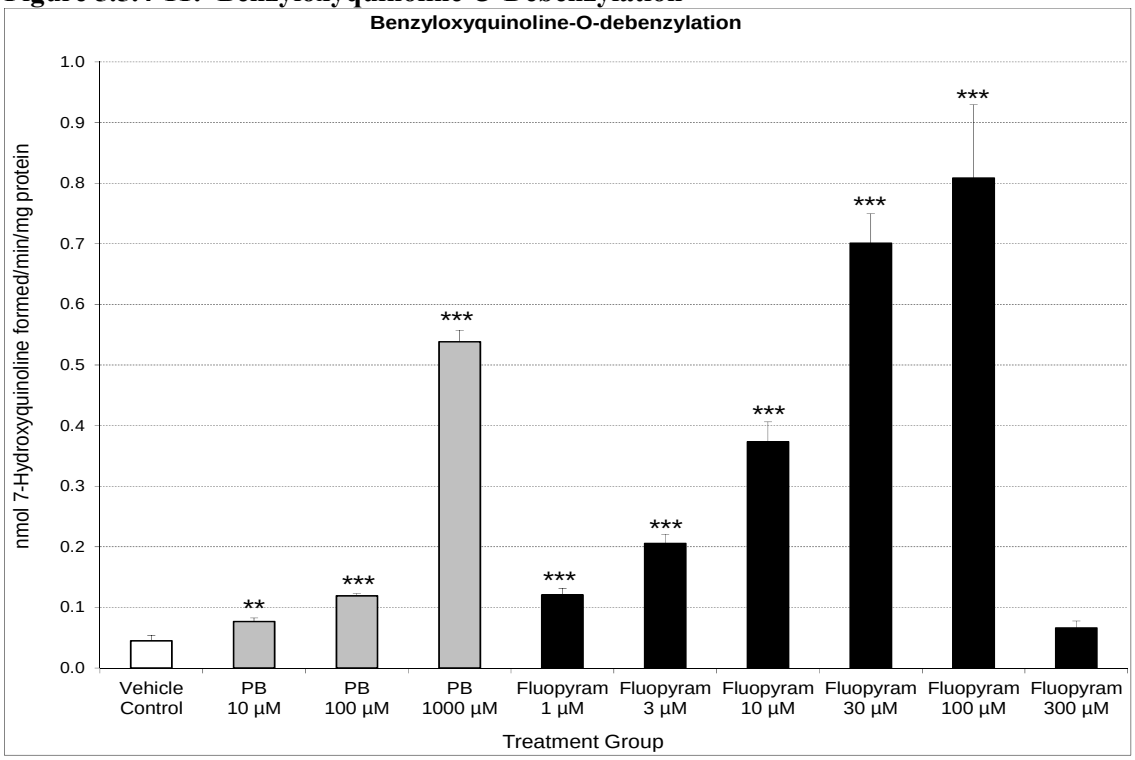
Culturing primary rat hepatocytes, for 96 hours, with phenobarbital resulted in a dose-dependent increase in BQ activity of up to 12-fold at 1000 µM. This is consistent with historical data. Exposure of hepatocytes to fluopyram resulted in a large, dose dependent increase in BQ activity of up to 18-fold at 100 µM.

**Table 5.5.4-51: Benzyloxyquinoline-*O*-Debenzylation**

| Treatment | BQ (nmol 7-Hydroxyquinoline formed/min/mg protein) |
|---|---|
| Vehicle Control | 0.045 ± 0.009 (100.0 ± 20.5)[a] |
| PB 10 µM | 0.077 ± 0.006** (170.8 ± 13.4) |
| PB 100 µM | 0.119 ± 0.004*** (265.5 ± 8.4) |
| PB 1000 µM | 0.538 ± 0.019*** (1198.8 ± 43.2) |
| Fluopyram 1 µM | 0.121 ± 0.011*** (268.7 ± 24.3) |
| Fluopyram 3 µM | 0.206 ± 0.015*** (457.9 ± 34.1) |
| Fluopyram 10 µM | 0.373 ± 0.033*** (831.1 ± 73.8) |
| Fluopyram 30 µM | 0.701 ± 0.048*** (1561.5 ± 107.8) |
| Fluopyram 100 µM | 0.808 ± 0.121*** (1800.9 ± 269.2) |
| Fluopyram 300 µM | 0.066 ± 0.011 (147.2 ± 25.5) |

[a] Values are Mean ± SD. Values in parenthesis are mean % control ± SD n = 3 per group. A Student's t-test (2-sided) was performed on the results; *statistically different from control p<0.05; ** p<0.01; ***p<0.001.

**Figure 5.5.4-11: Benzyloxyquinoline-*O*-Debenzylation**




## III. CONCLUSION

The clear increases in PROD activity seen after exposure to fluopyram indicate that fluopyram is an inducer of CYP2B. The induction of BROD and, particularly, BQ activity suggests that fluopyram is also a CYP3A inducer.

In common with phenobarbital, fluopyram stimulated replicative DNA synthesis (S-phase) in the hepatocytes.

In conclusion, these data suggest that fluopyram is an activator of both CAR and PXR.

**Supporting data regarding male mouse thyroid tumors**

In the mouse carcinogenicity study (see section 5.5.3), a higher incidence of thyroid follicular cell tumors was observed in male mice exposed to AE C656948 at 750 ppm (105 mg/kg bw/d) for 18 months compared to the corresponding controls. There were 7/50 animals affected in the high dose group compared to 1/50 in the control group. This finding was associated with a statistically significantly higher incidence of the pre-neoplastic lesion follicular cell hyperplasia. Retrospective examination of the thyroid gland of males sacrificed after 1 year of treatment revealed that 2/10 animals had thyroid follicular cell hyperplasia.

Thyroid effects were seen in association with marked liver effects at this dose level including centrilobular to panlobular hepatocellular hypertrophy, hepatocellular cholestasis, single cell degeneration/necrosis, interstitial mixed cell infiltrates, eosinophilic inclusion bodies and multinucleated hepatocytes. AE C656948 did not show any genotoxic potential (see section 5.4).

These data alone support the hypothesis of a non-genotoxic threshold mechanism for the thyroid follicular cell adenoma formation.

In addition, AE C656948 being a moderate phenobarbital-like cytochrome P-450 inducer in the rat (see section 5.3.1 and 5.5.4), one can hypothesize that an indirect mechanism of action for thyroid follicular cell adenoma, secondary to a liver enzyme induction in the mouse since the liver is heavily involved in the thyroid hormone metabolism through different enzymatic activities (Kelly, 2000[5]). In order to test this hypothesis, a series of studies were conducted including:

(1) an *in vitro* study on potential interaction of AE C656948 on thyroid peroxidase-catalysed reactions i.e. direct effect on thyroid hormone synthesis,

(2) an *in vivo* study to investigate the effects of AE C656948 on liver enzymes and on pituitary/thyroid hormone balance in the male mouse.

(3) two *in vivo* studies to investigate the effects of AE C656948 on the pharmacokinetics of intravenously administered $^{125}$I-thyroxine in the male mouse.

(4) an *in vivo* study to investigate the effects of AE C656948 on the expression of a selection of genes involved in the clearance of thyroid hormones and the detoxification of xenobiotics in the liver using qPCR analysis.

For the *in vivo* studies a similar investigation was performed using phenobarbital as a "positive control" reference compound for comparison. The same dose levels of AE C656948 and phenobarbital were used for each of the three investigations. A dose level of 2000 ppm AE C656948 was selected after evaluation of the results of a 28-day toxicity study in the mouse with this substance (see section 5.3.1), where 5000 ppm clearly exceeded the maximum tolerated dose and a 90-day toxicity study in the mouse (see section 5.3.2), where at the highest dose level tested, 1000 ppm, slight toxicity was observed. Therefore, the dose level of 2000 ppm was selected as an appropriate high dose level to be used in this study. Although, 2000 ppm was higher than the high dose used in the mouse carcinogenicity study (750 ppm), this dose level was selected to allow a clearer evaluation of the effects of AE C656948 without causing excessive toxicity. A dose level of 80 mg/kg/day phenobarbital was selected based on previous studies conducted with phenobarbital and was expected to provoke a positive response.

These studies are summarized hereafter.

The data generated indicate that

- AE C656948 did not affect the thyroid hormone synthesis at the thyroid peroxidase level supporting the absence of a direct effect on hormone synthesis.
- AE C656948 induced a similar hepatic enzymatic profile in male mice as phenobarbital i.e. induction of cytochrome P-450 content and specific induction of BROD and PROD activities.
- AE C656948 is able to modify the normal thyroid hormone levels in male mice, in particular by causing a decrease in T4 level associated with an increased level of TSH after only 3 days of treatment but also consistently after 2 weeks of treatment.
- AE C656948 induced a more rapid clearance of T4 from the blood than in the corresponding controls over the 24 hour period following $^{125}$I-thyroxine administration.

[5] Kelly G. (2000) Peripheral metabolism of thyroid hormones : a review. Alternative Medecine Review, Vol. 5 N°4, 306-333. KIIA 5.5.4 /10; Kelly, G.; 2000; M-300835-01

- AE C656948 induced an up-regulation of the sulfotransferase and UDP glucoronosyltransferase gene transcripts in the liver.
- Both AE C656948 and phenobarbital showed a similar pattern of effects in all *in-vivo* mouse mechanistic studies.

Overall, the data on AE C656948 support the hypothesis of a non-genotoxic indirect threshold mechanism for the formation of thyroid follicular cell adenomas in male mice. In particular, it was shown that when administered to male mice, AE C656948 was able to induce the metabolism capability of the liver and to cause a decrease in the T4 level which was associated with an increase in TSH levels. This increased level of TSH will stimulate the thyroid gland to produce thyroid hormones in order to restore their normal physiological levels and this constant stimulation at mid- or long-term is known to induce follicular cell hypertrophy/hyperplasia (observed in the mouse oncogenicity study already after 12 months in a few animals and clearly after 18 months in a large number of animals). In addition, the increased rate of clearance of T4 from the blood observed in AE C656948 treated animals was similar to that seen with the reference compound phenobarbital, which is known to induce microsomal β-D-glucuronyl transferases, which catalyse the metabolism of thyroxine to its glucuronide conjugate in the rodent (Bastomsky, 1973[6]). In the qPCR analysis assay, the gene transcrips in the liver that were up-regulated following treatment with AE C656948 and phenobarbital, i.e., the sulfotransferases and UDP glucoronosyltransferases, are known to encode for enzymes that inactivate T3 and T4 via glucoronide and sulfate derivatives.

In order to show a dose response for the key events leading to Car/Pxr-induced thyroid follicular cell tumors observed in male mice following life-time exposure to AE C656948 at the high dose level of 750 ppm, a series of 28-day feeding studies in the male mouse have been conducted using a range of dose levels which included dose levels used in the mouse carcinogenicity study, plus intermediate dose levels. The following parameters were assessed: specific cytochrome P-450 enzyme activities which acted as markers for activation of the Car/Pxr nuclear receptors and UDPGT species isoenzyme profiles, plasma levels of T4 and TSH, the gene expression of *Tsh* in the pituitary gland and thyroid follicular cell proliferation**.** Reversibility of changes seen following 28 days of treatment with AE C656948 was also assessed, phenobarbital was included as a "positive control" reference compound. A dose concordance was established for each key event and changes observed after up to 28 days of treatment with fluopyram were found to be reversible following cessation of treatment for 28 days.

In addition, a pivotal mechanistic study conducted to demonstrate Car/Pxr receptor activation as the first key event was a 28-day mouse study using both the wild-type (WT) C57BL/6J mouse and a genetically modified mouse that does not have functional Car or Pxr receptors (Pxr-Car KO), this study is summarized hereafter. Mice were exposed to AE C656948 at the tumorigenic dose (750 ppm) in the mouse cancer bioassay and above (1500 ppm), equivalent in terms of mg/kg/d to the rat tumorigenic dose level. <u>In this experiment, a significant induction of liver enzymes, liver enlargement and hepatocellular hypertrophy was seen in the WT mouse, but was not observed in the Pxr-Car KO mouse.</u>

[6] Bastomsky C.H. (1973) The biliary excretion of thyroxine and its glucoronic acid conjugate in normal and Gunn rats. Endocrinology., 92, 35-40. KIIA 5.5.4 /18;Bastomsky, C. H.;1973;M-308397-01

Other plausible modes of action (MoAs) for thyroid tumor formation have effectively been excluded.

The induction of chemical-specific thyroid tumors in rodents may be caused by the following MoAs (Capen, C.C. (1997). Mechanistic data and risk assessment of selected toxic end points of the thyroid gland. Toxicol. Pathol. 25(1):39-48: KIIA 5.5.4 /37;Capen, C. C.;1997;M-435031-01 and Hurley et al., 1998) Mode of carcinogenic action of pesticides inducing thyroid follicular cell tumors in rodents. Environ. Health Perspect. 106(8):437-45, KIIA 5.5.4 /38;Hurley, P. M.; Hill, R. N.; Whiting, R. J.;1998;M-086436-01.

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

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

Sex- and species differences

Although the basic functions of the hypothalamic-pituitary-thyroid axis are similar in animals and humans, long-term alteration of the pituitary-thyroid axis by chemicals in laboratory rodents (rats and mice) are more predisposed to give a higher incidence of hyperplasia and follicular cell tumors in those animals than in humans. Rats and mice are particularly sensitive to the decreased availability of T4 and T3 and respond with hypertrophy and hyperplasia of follicular cells and in long-term studies there is an increased incidence of thyroid tumors (Capen, 1992[7]). By contrast, humans are much less sensitive to elevated TSH levels or alteration of the thyroid function which are usually not associated with increase in thyroid cancer incidences (Hill *et al*. 1998[8]). For instance, there is no evidence that

[7] Capen C.C. (1992) Pathophysiology of chemical injury of the thyroid gland. Toxicology Letters., 64/65, 381-388. KIIA 5.5.4 /11; Capen, C. C.; 1998; M-300815-01

[8] Hill R.N., Crisp T.M., Hurley P.S., Rosenthal S.L. and Singh D.V. (1998) Risk assessment of thyroid follicular cell tumors. Environmental Health Perspestives, 106 (8), 447-457.
KIIA 5.5.4 /12; Hill, R. N.; Crisp, T. M.; Hurley, P. M.; Rosenthal, S. L.; Singh, D. V.; 1998; M-300844-01

chemicals such as phenobarbital, which affect thyroid function through a peripheral mechanism involving thyroid hormone metabolism, are associated with thyroid neoplasia in humans (McClain *et al.,* 1989[9]; Curran and De Groot, 1991[10]). The only verified cause of thyroid cancer in humans is exposure to ionizing radiation. The reasons for the interspecies difference in sensitivity are not completely known although one factor that probably plays a key role is the protein carriers of thyroid hormones. Both humans and rodents have nonspecific low affinity protein carrier of thyroid hormones (e.g. albumin). However, humans, other primates and dogs also have a high affinity binding protein (i.e. thyroxine-binding globulin) which binds preferably with T4 and which is missing in rodents and lower vertebrates. Hence, in rats, T4 is primarily bound to albumin and to a lesser extent to pre- and post-albumin. In mice, T4 is also bound preferably to albumin and postalbumin, whereas in humans T4 is bound to thyroxine binding globulin (van Raaij, 2002[11]). Consequently, the proportion of unbounded T4 (therefore more susceptible to metabolism and excretion) is greater in rodents than in humans (Capen, 1992[6]). In rodents, males have higher serum TSH levels than females and are usually more sensitive to goitrogenic stimulation and thyroid carcinogenesis (Chen, 1984[12]). In humans, there is no sex difference in hormone levels although females develop thyroid tumors more frequently than males (Boring *at al*., 1994[13]).

Overall, the mechanistic studies together with the standard repeat dose studies, clearly demonstrate the mode of action for the thyroid tumors in the mouse following a life-time exposure to fluopyram is mediated through and secondary to liver enzymes induction, *via* activation of the Car/Pxr nuclear receptors. The rodent specificity of liver mediated thyroid follicular cell tumors is well recognized and accepted as being non-relevant to humans.

Further detailed argumentation regarding mode of action and relevance to humans of the mouse thyroid tumors following exposure to fluopyram (AE C656948) are presented in the following Expert Summary documents:

Position Paper
Fluopyram: Mode of Action and Human Relevance Analysis of Rodent Liver and Thyroid Tumors MIIA Sec 3 /03; [redacted] 2013; M-465168-01

Expert Summary Report
Fluopyram: Mode of Action and Human Relevance Framework Analysis for Fluopyram-Induced Rodent Liver and Thyroid Tumors MIIA Sec 3 /02; [redacted] 2013; M-454439-01

[9] McClain R.M., Levin A.A., Posh R. and Downing J.C. (1989) The effect of phenobarbital on the metabolism and excretion of thyroxine in rats. Toxicology and Applied Pharmacology, 99, 216-228.
KIIA 5.5.4 /13; McClain, R. M.; Levin, A. A.; Posch, R.; Downing, J. C.; 1989; M-103760-01

[10] Curran P.G. and DeGroot L.J. (1991) The effect of hepatic enzyme-inducing drugs on thyroid hormones and the thyroid gland. Endocrine Reviews, 12 (2), 135-150.
KIIA 5.5.4 /14; Curran, P. G.; DeGroot, L. J.; 1991; M-066260-01

[11] Van Raaij M.T.M. (2002) Follicular cell thyroid tumors in rodents. In "Factsheets for the (eco)toxicological risk assessment strategy of the national institute for public health and the environment (RIVM), RIVM report 601516009, pp 27-42, Part II, editors Luttik, R.; Pelgrom, S. M. G. J.
KIIA 5.5.4 /15; Luttik, R.; Pelgrom, S. M. G. J.; 2002; M-300839-01

[12] Chen H.J. (1984) Age and sex differences in serum and pituitary thyrotropin concentrations in the rat : influence by pituitary adenoma. Experimental Gerontology, 19, 1-6.
KIIA 5.5.4 /16; Chen, H. J.; 1983; M-300821-01

[13] Boring C.C. (1994) Cancer statistics 1994. A Cancer Journal for Clinicians, 44, 7-26
KIIA 5.5.4 /17; Boring, C. C.; Squires, T. S.; Tong, T.; Montgomery, S.; 2008; M-300849-01

| | |
|---|---|
| **Report:** | KIIA 5.5.4 /03; [redacted] 2008; M-299276-01 |
| Title: | AE C656948 (Fluopyram) In vitro studies on the potential interactions with thyroid peroxidase-catalyzed reactions |
| Report No & | AT04481 |
| Document No | M-299276-01 |
| **Guidelines:** | **No specific guideline** |
| **Deviation** | **none** |
| **GLP** | **yes** |

**Executive 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 µM Amitrole for guaiacol oxidation and in the presence of 0.1 µM 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 µM, the highest concentration tested. Similarly, TPO-catalyzed iodine formation was not affected by 300 µM AE C656948.
These findings strongly suggest that AE C656948 does not affect thyroid hormone synthesis at the level of TPO.

## I. MATERIALS AND METHODS

- **MATERIALS:**

**1. Test Material:** AE C656948
**Description:** light beige powder
**Lot/Batch:** Mix-Batch:08528/0002
**Purity:** 94.7 %
**CAS:** 658066-35-4
**Stability of test compound:** Stable for a period covering the study duration

**2. Vehicle and /or positive control:** Dimethylsulfoxide

**3. Positive control substances:** Amitrole (3-amino-1,2,4-triazole) from Sigma (Lot number 083K0649)
ETU (ethylenethiourea, 2-imidazolidinethione) from Riedel-de-Haën (Lot number 3223X)

**4. Biological raw material** Hog thyroid glands from domestic pigs were obtained from Bayer CropScience, [redacted]. They were trimmed free of excess fat and connective tissue. They were stored at -80°C until use.

- **STUDY DESIGN:**

**1. Experimental dates:** The study was conducted on October 4, 2007

**2. Microsome preparation** Interactions of AE C656948 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 Solubilized hog thyroid microsomes were prepared according to a standard procedure as described in [redacted] 1984

**3. Statistics** No statistical evaluation was performed

**B. METHODS:**

**1. Determination of TPO-catalyzed guaiacol oxidation**

Guaiacol oxidation was used as a measure for peroxidative activity. Incubations were carried out at room temperature in 0.1 M potassium phosphate buffer, pH 7.4 in a total volume of 1.0 mL. Guaiacol (125 µL of 40 mM solution in water, final concentration 5 mM), TPO (approximately 0.1 ΔE/min, corresponds to 3.5 µL of microsomal preparation) and test compound were preincubated for 1 minute, then the reaction was initiated by addition of hydrogen peroxide (20 µL of 12.5 mM solution in water, final concentration 250 µM). Test compounds were added in 20 µL DMSO, likewise control incubations lacking test compounds contained the same amount of solvent.

The following final concentrations were used:

AE C656948: 3.0 – 30 – 300 µM
Amitrole: 1.0 µM

The initial linear increase (ΔE/min) of the absorption at 470 nm was used to calculate the peroxidase activity.

**2. Determination of TPO-catalyzed iodine formation**

Incubations were carried out as described above, however, guaiacol was replaced by potassium iodide (100 µL of 100 mM solution in water, final concentration 10 mM).

The following final concentrations were used:

AE C656948: 3.0 – 30 – 300 µM
Amitrole: 0.1 µM
ETU: 5 µM

The initial linear increase (ΔE/min) of the absorption at 350 nm was used to calculate the enzymatic activity.

## II. RESULTS AND DISCUSSION

The results of the TPO-catalyzed oxidation of guaiacol are summarized in Table 5.5.4-9. Amitrole, the positive control, at a concentration of 1 µM inhibited the initial rate of thyroid peroxidase (TPO)-catalyzed oxidation of the model substrate guaiacol by more than 50%. AE C656948, at concentrations up to 300 µM did not affect this reaction.

**Table 5.5.4-52: Effect of AE C656948 on TPO-catalyzed guaiacol reaction**

| Compound | Concentration (µM) | ΔE/min. ± SD | % of control |
|---|---|---|---|
| Vehicle | - | 0.121 ± 0.006 | 100 |
| AE C656948 | 3 | 0.122 ± 0.002 | 100.8 |
| | 30 | 0.123 ± 0.005 | 101.6 |
| | 300 | 0.124 ± 0.001 | 102.5 |
| Amitrole | 1 | 0.054 ± 0.003 | 44.6 |

The results of the TPO-catalyzed iodine formation are summarized in Table 5.5.4-10. Up to 300 µM AE C656948 did not affect TPO-catalyzed iodine formation. Neither the initial rate of the reaction was affected, or a temporary suppression of iodine formation was observed. In contrast, Ethylenethiourea (ETU), a trap of the iodinating intermediate, temporarily suppressed iodine formation, whilst Amitrole at a concentration of 0.1 µM inhibited the initial rate of this reaction by 50%.

**Table 5.5.4-53: Effect of AE C656948 on TPO-catalyzed iodine formation**

| Compound | Concentration (µM) | ΔE/min. ± SD | % of control |
|---|---|---|---|
| Vehicle | - | 0.259 ± 0.012 | 100 |
| AE C656948 | 3 | 0.269 ± 0.012 | 103.9 |
| | 30 | 0.246 ± 0.005 | 95.0 |
| | 300 | 0.260 ± 0.012 | 100.4 |
| Amitrole | 0.1 | 0.131 ± 0.013 | 50.6 |

## III. CONCLUSION

AE C656948, at concentrations up to 300 µM (the highest concentration tested) neither affected TPO-catalyzed guaiacol oxidation nor iodine formation. These findings strongly suggest that AE C656948 does not affect thyroid hormone synthesis at the level of TPO.

| | |
|---|---|
| **Report:** | KIIA 5.5.4 /04; [redacted] 2008; M-299522-01 |
| Title: | AE C656948 Mechanistic 14-day toxicity study in the mouse by dietary administration (hepatotoxicity and thyroid hormone investigations) |
| Report No & | SA 07215 |
| Document No | M-299522-01 |
| **Guidelines:** | **No specific guideline** |
| **Deviations:** | **none** |
| **GLP** | **yes** |

**Executive summary**

AE C656948 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 were 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 level measurements (T3, T4 and TSH). The liver and thyroid gland were weighed and sampled for microscopic examination. In addition, hepatic cytochrome P-450 isoenzymes and UDPGT activities were also measured using 4-nitrophenol as substrate.

After 3 days of exposure, at 2000 ppm, mean T3 level was not changed when compared to the untreated control group, whereas mean T4 level was decreased (-30%) while at the same time, 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 observed in all examined treated animals. In addition, hepatocellular single cell necrosis was observed in 1/5 treated animals. No significant microscopic change was observed in the thyroid gland.

Total cytochrome P-450 content was markedly increased (+116%) by the treatment. EROD activities were marginally increased (+235%), whereas PROD and BROD activities were markedly increased (respectively +2890% and +8717%) when compared to controls. No significant changes were observed in UDPGT.

After 14 days of exposure at 2000 ppm, overall the results were very similar to those obtained after 3 days of exposure. Mean T3 level was not changed whereas mean T4 level was still decreased (-27%) and mean TSH level was still increased (+7%), when compared to controls. Mean absolute and relative liver weights were increased by approximately 60% when compared to the control animals. At macroscopic observation, enlarged liver was found in 13/15 treated animals, compared to no incidences in the controls. Furthermore, dark liver was observed in 14/15 treated animals compared to only 1 in the control animals. At microscopic examination, diffuse centrilobular to panlobular hepatocellular hypertrophy was seen in all examined treated animals. In addition, hepatocellular single cell necrosis was observed in 4/5 treated animals. 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 (equivalent to between 308 and 314 mg/kg bw/day) in the C57BL/6J mouse for 3 and 14 days induced a phenobarbital-like P-450 hepatic enzymatic activity profile and has the ability to modify the normal thyroid hormone balance (in particular to cause a sustainable decrease in T4 level and increase in TSH level), which in turn can indirectly induce thyroid changes.

# I. MATERIALS AND METHODS

## A. MATERIALS:

**Test Material:** AE C656948
**Description:** Light beige powder
**Lot/Batch:** Mix-Batch:08528/0002
**Purity:** 94.7 %
**CAS:** 658066-35-4
**Stability of test compound:** Stable in rodent diet for a period covering the study duration

**2. Vehicle and /or positive control:** None

**3. Test animals:**
**Species**: Mouse – Male only
**Strain:** C57BL/6J
**Age:** 8 weeks approximately at start of treatment
**Weight at dosing:** 21.2 to 25.1 g
**Acclimation period:** 5 to 6 days
**Diet:** Certified rodent powdered and irradiated diet A04C-10 P1 from [redacted] ([redacted], [redacted] France), *ad libitum*
**Water:** Tap water, *ad libitum*
**Housing:** Animals were caged individually in suspended stainless steel wire mesh cages.
**Environmental conditions –**
**Temperature:** 20 – 24°C
**Humidity:** 40 – 70 %
**Air changes:** Approximately 10-15 changes per hour
**Photoperiod:** Alternating 12-hour light and dark cycles (7 am- 7 pm)

**B. STUDY DESIGN:**

**1. In life dates:** Dosing period from October 1 to 4, 2007 for the 3-day exposure groups and from September 18 to October 2, 2007 for the 14-day exposure groups

**2. Animal assignment and treatment**

The dose level was set after evaluation of the results of a subacute toxicity study in the mouse with this substance (see section 5.3.1). In this study, 5000 ppm clearly exceeded the maximum tolerated dose as all males and most females had to be sacrificed for humane reasons before the end of the study. Therefore, the dose level of 2000 ppm was selected as an appropriate high dose level to be used in this study. Although, 2000 ppm was higher than the high dose used in the mouse carcinogenicity study (750 ppm), this dose level was selected to allow a clearer evaluation of the effects of AE C656948 without causing excessive toxicity. Males only were used in this study as an increased incidence of thyroid follicular cell adenoma was observed in this sex only.
Animal housing and husbandry were in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals (Public Health Service, National Institute of Health, NIH publication N°86-23, revised 1985) and "Le Guide du Journal Officiel des Communautés Européennes L358, 18 Décembre 1986, N°86/609/CEE du 24 Novembre 1986".

**Table 5.5.4-54: Study design**

| Test group | Treatment | Dose level in ppm (mg/kg bw/day) | Duration of treament | Animals assigned |
|---|---|---|---|---|
| 1 | Control | 0 | 3 days | 15 |
| 2 | AE C656948 | 2000 (308) | 3 days | 15 |
| 3 | Control | 0 | 14 days | 15 |
| 4 | AE C656948 | 2000 (314) | 14 days | 15 |

The stability of the test substance in the diet at 20 and 10000 ppm has been demonstrated in a previous study after a freezing period of 95 days followed by storage periods at room temperature of 10 days or 105 days. Before the start of the study homogeneity and concentration were checked on the study mix at 2000 ppm. The mean value obtained from the homogeneity check was taken as measured concentration. The study mix was found to be acceptable for use on the study as the homogeneity and concentration checks were within the range of 102 to 103% of the nominal concentration.

**3. Diet and water**

Certified rodent powdered and irradiated diet A04CP1-10 from [redacted] ([redacted] France) and filtered and softened tap water from the municipal water supply in individual bottles were available *ad libitum*, except prior to sacrifice when animals were diet fasted overnight. Routine analyses of food and water indicated that there was no contamination which could have compromised the study

**4. Statistics**

- – Body weight change parameters,
  – Terminal body weight, absolute and relative organ weight parameters,

Mean and standard deviation were calculated for each group and per time period for body weight change parameters.
The F test was performed to compare the homogeneity of group variances.
If the F test was not significant ($p>0.05$), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided).
If the F test was significant ($p\leq0.05$), mean of the exposed group was compared to the mean of the control group using the modified t-test (2-sided).

- – Body weight and average food consumption/day parameters
  – Total cytochrome P450 content
  – Hormonal parameters

Mean and standard deviation were calculated for each group and per time period for average food consumption/day parameters.
The F test was performed to compare the homogeneity of group variances.
If the F test was not significant ($p>0.05$), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided).
If the F test was significant ($p\leq0.05$), data were transformed using the log transformation. If the F test on log transformed data was not significant ($p>0.05$), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided) on log transformed data.
If the F test was significant ($p\leq0.05$) even after log transformation, the mean of the exposed group was compared to the mean of the control group using the modified t-test (2-sided).
If one or more group variance(s) equal 0, means were compared using non-parametric procedures.

- - Enzymatic activities and cell proliferation parameter

Mean of the exposed group will be compared to the mean of the control group using the exact Mann-Whitney test (2-sided).

Group means will be compared at the 5% and 1% levels of significance.
Statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics) except for liver enzyme parameters and cell proliferation parameters which were analyzed using SAS programs version 8.2.

## C. METHODS:

### 1. Observations

The animals were observed twice daily for moribundity and mortality (once daily on weekends or public holidays). Observed clinical signs were recorded at least once daily for all animals and detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility, and duration of any clinical signs were recorded.

### 2. Body weight

Each animal was weighed at least once during the acclimatization phase, on study Day 1 and then weekly during the treatment period. Additionally, diet fasted animals were weighed before scheduled necropsy.

### 3. Food consumption

The weight of food supplied and of that remaining at the end of the food consumption period was recorded weekly for all animals during the treatment period to calculate the mean daily food consumption. Any food spillage was noted.

### 4. Hormonal investigation

**Blood sampling**
On study Day 4 (Groups 1 and 2) and on study Day 15 (Groups 3 and 4), blood samples were taken from all animals by puncture of the retro-orbital venous plexus. Animals were anesthetized by inhalation of Isoflurane ([redacted], France) prior to bleeding. Blood was collected on lithium heparin for hormone level evaluation.

**Hormone measurements**
Plasma was prepared from each blood sample and kept frozen at approximately -80°C until the determination of TSH, T3 and T4 hormone levels with specific radio-immunoassay kits (supplied by Amersham for TSH and by DIASORIN for T3 and T4).

### 5. Sacrifice and pathology

**Necropsy procedure – Organ sampling**
On study Day 4 and on study day 15, all animals from groups 1 and 2 and from groups 3 and 4, respectively were sacrificed by exsanguination under deep anesthesia by inhalation of Isoflurane ([redacted], France). All animals were necropsied. The necropsy included the examination of the external surfaces, all orifices and all major organs, tissues and body cavities. Macroscopic abnormalities were recorded but not sampled except upon decision of the Study Pathologist. Brain and liver were weighed fresh.
A piece of median and left lobe liver of 5 animals as well as the thyroid gland (with parathyroid glands) of all the animals were sampled and fixed in 10% neutral buffered formalin for histological examination. The remaining portions of livers from those 5 animals and the entire liver of the other 10 animals of each group were used for microsome preparation.

**Histotechnology - Histopathology**
Liver portions and thyroid glands sampled for microscopy were embedded in paraffin wax. Histological sections, stained with hematoxylin and eosin, were prepared and examined

**6. Hepatotoxicity testing:**

At scheduled sacrifice dates, in each group the livers of 3 animals were pooled (each time 2 entire livers and the remaining portion of 1 liver used for histological examination) and homogenized for microsomal preparations. In total, 5 microsomal samples per group were prepared. Total cytochrome P-450 content and specific cytochrome P-450 isozyme profile (including EROD, BROD and PROD activities) were measured to check the hepatotoxic potential of the test substance. In addition, Phase II enzymatic activities were also determined by measuring UDP-glucuronosyltransferase (UDPGT) with 4-nitrophenol as substrate.

## II. RESULTS AND DISCUSSION

### A. OBSERVATIONS:

Mortality:

There was no mortality in any groups throughout the study.

Clinical signs:

There were no treatment-related clinical signs.

### B. BODY WEIGHT AND BODY WEIGHT GAIN:

There was no effect on body weights or body weight gains throughout the study.

### C. FOOD CONSUMPTION:

There was a slight reduction in food consumption at the beginning of treatment with AE C656948 (-12.5% at the end of the 3-day exposure period and -5.1% after 1 week in the 14-day exposure period). Thereafter, food consumption was comparable to controls.

## D. HORMONE ANALYSIS

The results of TSH, T3 and T4 levels are summarized in Table 5.5.4-12.
The results are very comparable between the 2 time-points in both cases, while T3 levels remained unaffected, a significant decrease in T4 levels (-30 and -27%, respectively) together with a significant increase in TSH levels (+18 and +7%, respectively) was seen in treated groups compared to control groups. These results are consistent with the known feedback regulation mechanism of thyroid hormones where when thyroid hormones levels are low, the pituitary gland will produce TSH to stimulate the thyroid gland in order to restore the normal level of thyroid hormones.

**Table 5.5.4-55: Mean levels of thyroid/pituitary hormone after 3 and 14 days of exposure to AE C656948**

| | 3-day exposure | | 14-day exposure | |
|---|---|---|---|---|
| **Group** | **Control** | **2000 ppm (308 mg/kg bw/d)** | **Control** | **2000 ppm (314 mg/kg bw/d)** |
| T3 (nmol/L) | 1.62 ± 0.15 | 1.64 ± 0.25 (+1%) | 1.45 ± 0.18 | 1.52 ± 0.38 (+5%) |
| T4 (nmol/L) | 43.7 ± 8.1 | 30.7** ± 6.0 (-30%) | 38.1 ± 9.1 | 27.7** ± 8.7 (-27%) |
| TSH (ng/mL) | 3.81 ± 0.23 | 4.48** ± 0.31 (+18%) | 3.81 ± 0.28 | 4.09* ± 0.44 (+7%) |

*: p≤0.05; **: p≤0.01

## E. : SACRIFICE AND PATHOLOGY

As seen in other studies in the mouse (see section 5.3), liver weights were markedly increased in the treated group compared to control groups after only 3 days of exposure and after 14 days of exposure (approximately +60% in all cases). This was associated with enlarged and/or dark liver seen at the macroscopic examination and with hepatocellular hypertrophy in all examined animals and single cell necrosis on many occasions especially after 14 day of exposure. An increased number of mitoses was also observed in all animals after 3 days of exposure.

**Table 5.5.4-56: Pathological liver effects after 3 and 14 days of exposure to AE C656948**

| | 3-day exposure | | 14-day exposure | |
|---|---|---|---|---|
| **Group** | **Control** | **2000 ppm (308 mg/kg bw/d)** | **Control** | **2000 ppm (314 mg/kg bw/d)** |
| **Liver weights** | | | | |
| Absolute weight (g) | 1.24±0.12 | 1.97±0.17** (+59%) | 1.25±0.10 | 1.99±0.22** (+59%) |
| Body weight ratio (%) | 5.40±0.57 | 8.71±0.54** (+61%) | 5.23±0.33 | 8.42±0.94** (+61%) |
| Brain weight ratio (%) | 279.99±29.24 | 448.34±34.51** (+60%) | 286.49±21.42 | 462.30±52.04** (+61%) |
| **Histopathological findings** | | | | |
| Centrilobular to panlobular hepatocellular hypertrophy | 0/5 | 5/5 | 0/5 | 5/5 |
| Increase mitosis | 0/5 | 5/5 | 0/5 | 0/5 |
| Single cell necrosis | 0/5 | 1/5 | 0/5 | 4/5 |

*: p≤0.05; **: p≤0.01

No relevant changes were observed in the thyroid gland.

**Hepatotoxicity testing**

The results of hepatotoxicity testing are described in Table 5.5.4-14.

At both time points, treatment with AE C656948 induced a clear increase in total cytochrome P-450 content and a marked increase in BROD and PROD activities compared to controls. EROD activity was only slightly increased whereas 4-nitrophenol UDPGT activity was unaffected. These results are very comparable with those obtained with phenobarbital with the same study design (see following study). They are also quite similar to the changes observed in the rat with the exception of the 4-nitrophenol UDPGT activity which was also induced in the rat but was not affected in the mouse.

**Table 5.5.4-57: Cytochrome P-450 content and enzymatic activities in the liver after 3 and 14 days of exposure to AE C656948**

| | 3-day exposure | | 14-day exposure | |
|---|---|---|---|---|
| **Group** | **Control** | **2000 ppm (308 mg/kg bw/d)** | **Control** | **2000 ppm (314 mg/kg bw/d)** |
| P-450 (nmol/mg Prot.) | 1.08 ± 0.16 | 2.33 ± 0.19 (+116%) ** | 1.26 ± 0.49 | 2.15 ± 0.06 (+71%) * |
| EROD (pmol/min/ mg Prot.) | 90.25 ± 11.11 | 302.52 ± 43.49 (+235%) ** | 99.05 ± 8.98 | 262.24 ± 72.87 (+165%) ** |
| PROD (pmol/min/ mg Prot.) | 4.93 ± 0.83 | 143.42 ± 57.05 (+2890%) ** | 4.19 ± 0.49 | 94.80 ± 44.77 (+2163%) ** |
| BROD (pmol/min/ mg Prot.) | 12.99 ± 2.34 | 1145.28 ± 262.93 (+8717%) ** | 12.83 ± 2.11 | 1175.30 ± 163.99 (+9061%) ** |
| UDPGT (nmol/min/ mg Prot.) | 16.04 ± 1.42 | 15.36 ± 0.53 (N.C.) | 17.09 ± 0.90 | 14.32 ± 0.63 (-16%) ** |

*: p≤0.05; **: p≤0.01

## III. CONCLUSION

These data indicate that AE C656948 has the ability in male C57BL/6J mice to:
- modify the thyroid hormone balance by causing a decrease in T4 levels and a concomitant increase in TSH,
- induce liver changes including in particular an induction of phenobarbital-like hepatic enzymes including total cytochrome P-450, PROD, BROD activities.

When compared to the results obtained with phenobarbital using the same study design, these results also indicate a good similarity of effects between AE C656948 and phenobarbital.

| | |
|---|---|
| **Report:** | KIIA 5.5.4 /05; [redacted] 2008; M-299521-01 |
| Title: | Phenobarbital mechanistic 14-day toxicity study in the mouse by oral gavage (hepatotoxicity and thyroid hormone investigations) |
| Report No & | SA 07326 |
| Document No | M-299521-01 |
| **Guidelines:** | **No specific guideline** |
| **Deviations:** | **none** |
| **GLP** | **yes** |

**Executive summary**

Phenobarbital, a reference hepatotoxic 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 were 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.
After 3 days of exposure, 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 by between 4% to 11% compared to the control group. This was associated with macroscopic findings including enlarged and/or dark liver in several animals and with microscopic observations including diffuse centrilobular to panlobular hepatocellular hypertrophy in most animals.
Total cytochrome P-450 content was markedly 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, the mean T3 level was not changed whereas the mean T4 level was statistically significantly decreased (-19%) and the mean TSH level was statistically significantly increased (+9%) when compared to controls. Effects in the liver were very comparable after 14 days of exposure than after 3 days of exposure. At necropsy mean absolute and relative liver weights were increased by between 21% to 23% compared to the control group. At macroscopic observation, enlarged liver was found in most treated animals (12/15) and dark liver was observed in 4/15 treated animals. At microscopic examination, diffuse centrilobular to panlobular hepatocellular hypertrophy were seen in all treated animal.
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 (+1345% and 2844%, respectively) when compared to controls. No significant change was observed in UDPGT activities when measured with 4-nitrophenol as substrate.

In conclusion, this study demonstrated 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 significant liver changes including increases in hepatic total cytochrome P-450, PROD and BROD activities. In addition, treatment with phenobarbital was associated with a modification of the normal thyroid hormone balance (in particular by a decrease in T4 and/or T3 after 3 and/or 14 days of exposure together with a concomitant increase in TSH level after 14 days.

# I. MATERIALS AND METHODS

## A. MATERIALS:

**1. Test Material:** Phenobarbital
**Description:** White crystalline powder
**Lot/Batch:** 06100228
**Purity:** 99.6 %
**CAS:** 50-06-6
**Stability of test compound:** Stable for a period covering the study duration

**2. Vehicle and /or positive control:** Methylcellulose 400

**3. Test animals:**
**Species**: Mouse – Male only
**Strain:** C57BL/6J
**Age:** 8 weeks approximately at start of treatment
**Weight at dosing:** 19.9 to 24.2 g
**Acclimation period:** 6 days
**Diet:** Certified rodent powdered and irradiated diet A04C-10 P1 from [redacted] ([redacted], [redacted], France), *ad libitum*
**Water:** Tap water, *ad libitum*
**Housing:** Animals were caged individually in suspended stainless steel wire mesh cages.
**Environmental conditions –**
**Temperature:** 20 – 24°C
**Humidity:** 40 – 70 %
**Air changes:** Approximately 10-15 changes per hour
**Photoperiod:** Alternating 12-hour light and dark cycles (7 am- 7 pm)

## B. STUDY DESIGN:

**1. In life dates:** Dosing period from November 27 to 30, 2007 for the 3-day exposure groups and from November 20 to December 4, 2007 for the 14-day exposure groups

**2. Animal assignment and treatment**

Phenobarbital was administered once daily for 3 or 14 days by oral gavage to groups of 15 males at the dose of 80 mg/kg/day in 0.5% aqueous solution of methycellulose 400. The dose level was selected on the basis of previous studies conducted with phenobarbital. Negative control groups with the same number of animals received the vehicle only (0.5% aqueous solution of methycellulose 400). Animal housing and husbandry were in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals (Public Health Service, National Institute of Health, NIH publication N°86-23, revised 1985) and "Le Guide du Journal Officiel des Communautés Européennes L358, 18 Décembre 1986, N°86/609/CEE du 24 Novembre 1986".

**Table 5.5.4-58: Study design**

| Test group | Treatment | Dose level (mg/kg bw/day) | Duration of treament | Animals assigned |
|---|---|---|---|---|
| 1 | Control | 0 | 3 days | 15 |
| 2 | Phenobarbital | 80 | 3 days | 15 |
| 3 | Control | 0 | 14 days | 15 |
| 4 | Phenobarbital | 80 | 14 days | 15 |

Before the start of the study homogeneity and concentration were checked on the test formulation at 8 g/L. The mean value obtained from the homogeneity check was taken as measured concentration. The results of homogeneity and concentration of phenobarbital in the test formulation were found to be acceptable, being within a range of between 97% and 99% of the nominal concentration.

**3. Diet and water**

Certified rodent powdered and irradiated diet A04CP1-10 from [redacted] ([redacted], France) and filtered and softened tap water from the municipal water supply in individual bottles were available ad libitum, except prior to sacrifice when animals were diet fasted overnight. Routine analyses of food and water indicated that there was no contamination which could have compromised the study

**4. Statistics**

- Body weight change parameters,
- Terminal body weight, absolute and relative organ weight parameters,

Mean and standard deviation were calculated for each group and per time period for body weight change parameters.
The F test was performed to compare the homogeneity of group variances.
If the F test was not significant ($p>0.05$), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided).
If the F test was significant ($p\leq0.05$), mean of the exposed group was compared to the mean of the control group using the modified t-test (2-sided).

- Body weight and average food consumption/day parameters
- Total cytochrome P450 content
- Hormonal parameters

Mean and standard deviation were calculated for each group and per time period for average food consumption/day parameters.
The F test was performed to compare the homogeneity of group variances.
If the F test was not significant ($p>0.05$), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided).
If the F test was significant ($p\leq0.05$), data were transformed using the log transformation. If the F test on log transformed data was not significant ($p>0.05$), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided) on log transformed data.
If the F test was significant ($p\leq0.05$) even after log transformation, mean of the exposed group was compared to the mean of the control group using the modified t-test (2-sided).
If one or more group variance(s) equal 0, means were compared using non-parametric procedures.

- - Enzymatic activities and cell proliferation parameter

Mean of the exposed group will be compared to the mean of the control group using the exact Mann-Whitney test (2-sided).

Group means will be compared at the 5% and 1% levels of significance.
Statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics) except for liver enzyme parameters and cell proliferation parameters which were analyzed using SAS programs version 8.2.

## C. METHODS:

### 1. Observations

The animals were observed twice daily for moribundity and mortality (once daily on weekends or public holidays). Observed clinical signs were recorded at least once daily for all animals and detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility, and duration of any clinical signs were recorded.

### 2. Body weight

Each animal was weighed at least once during the acclimatization phase, on study Day 1 and then weekly during the treatment period. Additionally, diet fasted animals were weighed before scheduled necropsy.

### 3. Food consumption

The weight of food supplied and of that remaining at the end of the food consumption period was recorded weekly for all animals during the treatment period. Any food spillage was noted.

### 4. Hormonal investigation

**Blood sampling**
On study Day 4 (Groups 1 and 2) and on study Day 15 (Groups 3 and 4), blood samples were taken from all animals by puncture of the retro-orbital venous plexus. Animals were anesthetized by inhalation of Isoflurane ([redacted], France) prior to bleeding. Blood was collected on lithium heparin for hormone level evaluation.

**Hormone measurements**
Plasma was prepared from each blood sample and kept frozen at approximately -80°C until the determination of TSH, T3 and T4 hormone levels with specific radio-immunoassay kits (supplied by Amersham for TSH and by DIASORIN for T3 and T4).

### 5. Sacrifice and pathology

**Necropsy procedure – Organ sampling**
On study Day 4 and on study day 15, all animals from groups 1 and 2 and from groups 3 and 4, respectively were sacrificed by exsanguination under deep anesthesia by inhalation of Isoflurane ([redacted] France). All animals were necropsied. The necropsy included the examination of the external surfaces, all orifices and all major organs, tissues and body cavities. Macroscopic abnormalities were recorded but not sampled except upon decision of the Study Pathologist. Brain and liver were weighed fresh.
A piece of median and left lobe liver of 5 animals as well as the thyroid gland (with parathyroid glands) of all the animals were sampled and fixed in 10% neutral buffered formalin for histological examination. The remaining portions of livers were used for microsome preparation.

**Histotechnology - Histopathology**
Liver portions and thyroid glands sampled for microscopy were embedded in paraffin wax. Histological sections, stained with hematoxylin and eosin, were prepared and examined

**6. Hepatotoxicity testing:**

At scheduled sacrifice dates, in each group the livers of 3 animals were pooled (each time 2 entire livers and the remaining portion of 1 liver used for histological examination) and homogenized for microsomal preparations. In total, 5 microsomal samples per group were prepared. Total cytochrome P-450 content and specific cytochrome P-450 isozyme profile (including EROD, BROD and PROD activities) were measured to check the hepatotoxic potential of the test substance.
In addition, Phase II enzymatic activities were also determined by measuring UDP-glucuronosyltransferase (UDPGT) with 4-nitrophenol as substrate.

## II. RESULTS AND DISCUSSION

### A. OBSERVATIONS:

Mortality:

There was no mortality in any groups throughout the study.

Clinical signs:

There were no treatment-related clinical signs.

### B. BODY WEIGHT AND BODY WEIGHT GAIN:

The first days of exposure were associated with lower body weight gains in treated groups compared to controls. For the 3-day exposure group, an overall mean body weight loss of 0.6 g was observed at the end of treatment compared to a gain of 0.3 g in the control group. In the 14-day exposure group, there was a mean body weight loss of 0.3 g ($p \leq 0.05$) in the treated group compared with a gain of 0.4 g in the control group on study Day 7 although overall mean bodyweight was not affected at the end of treatment period.

### C. FOOD CONSUMPTIONS:

There was a slight reduction in food consumption at the beginning of treatment but not during the second week of treatment.

### D. HORMONE ANALYSIS

The results of TSH, T3 and T4 levels are summarized in Table 5.5.4-16.
After 3 day of exposure, statistically significantly lower levels of both T3 and T4 were observed in the treated group compared to control while TSH level remained unaffected.
After 14 days of exposure T4 level was still significantly lower in the treated group than in the control with a concomitant significant higher level of TSH. An increased level of TSH is expected when T4 level is low as TSH will stimulate thyroid to produce T4.

**Table 5.5.4-59: Mean levels of thyroid/pituitary hormone after 3 and 14 days of exposure to phenobarbital**

| | 3-day exposure | | 14-day exposure | |
|---|---|---|---|---|
| **Group** | **1 Control** | **2 Phenobarbital** | **3 Control** | **4 Phenobarbital** |
| T3 (nmol/L) | 1.72 ± 0.25 | 1.54* ± 0.17 (-10%) | 1.61 ± 0.20 | 1.57 ± 0.19 (-2%) |
| T4 (nmol/L) | 36.7 ± 6.2 | 26.8** ± 3.5 (-27%) | 32.4 ± 6.5 | 26.1* ± 6.7 (-19%) |
| TSH (ng/mL) | 4.44 ± 0.27 | 4.41 ± 0.54 (-1%) | 4.47 ± 0.37 | 4.89* ± 0.58 (+9%) |

*: p≤0.05; **: p≤0.01

## E. : SACRIFICE AND PATHOLOGY

Higher liver weights were observed in animals treated with phenobarbital compared to controls after both 3 and 14 days of exposure. After 14-day of treatment, absolute and relative liver weight were higher (+21 to 23%) whereas after 3 day of treatment, only liver to bodyweight ratio was significantly higher (+11%) compared to control. This was associated in several occasions with enlarged and/or dark liver observed at the macroscopic examination and with hepatocellular hypertrophy at the microscopic examination.

**Table 5.5.4-60: Pathological liver effects after 3 and 14 days of exposure to phenobarbital**

| | 3-day exposure | | 14-day exposure | |
|---|---|---|---|---|
| **Group** | **1 Control** | **2 Phenobarbital** | **3 Control** | **4 Phenobarbital** |
| **Liver weights** | | | | |
| Absolute weight (g) | 1.30±0.12 | 1.36±0.09 (+5%) | 1.31±0.16 | 1.60**±0.16 (+22%) |
| Body weight ratio (%) | 5.70±0.33 | 6.32**±0.21 (+11%) | 5.38±0.50 | 6.65**±0.47 (+23%) |
| Brain weight ratio (%) | 307.79±34.56 | 320.20±22.02 (+4%) | 301.33±34.16 | 365.46**±47.51 (+21%) |
| **Histopathological findings** | | | | |
| Centrilobular to panlobular hepatocellular hypertrophy | 0/5 | 4/5 | 0/5 | 5/5 |
| Increased mitosis | 0/5 | 3/5 | 0/5 | 0/5 |

*: p≤0.05; **: p≤0.01

**Hepatotoxicity testing**

The results are described in Table 5.5.4-18. At both time points, treatment with phenobarbital induced clear increase in total cytochrome P-450 content and a marked increase in BROD and PROD activities compared to controls. EROD activity was only slightly increased whereas 4-nitrophenol UDPGT activity was unaffected. Those results are in line the changes observed in the rat with the exception of the 4-nitrophenol UDPGT activity which was also induced in the rat.

**Table 5.5.4-61: Cytochrome P-450 content and enzymatic activities in the liver after 3 and 14 days of exposure to phenobarbital**

| | 3-day exposure | | 14-day exposure | |
|---|---|---|---|---|
| **Group** | **1**<br>**Control** | **2**<br>**Phenobarbital** | **3**<br>**Control** | **4**<br>**Phenobarbital** |
| P-450<br>(nmol/mg Prot.) | 0.94 ± 0.06 | 2.31**± 0.20<br>(+ 146%) | 0.98 ± 0.12 | 1.33* ± 0.29<br>(+ 36%) |
| EROD<br>(pmol/min/ mg Prot.) | 48.08 ± 6.87 | 190.65** ± 97.94<br>(+ 297%) | 35.34 ± 10.48 | 167.88** ± 96.47<br>(+ 375%) |
| PROD<br>(pmol/min/ mg Prot.) | 6.01 ± 1.99 | 88.99** ± 29.89<br>(+ 1381%) | 4.98 ± 0.59 | 71.97** ± 21.55<br>(+ 1345%) |
| BROD<br>(pmol/min/ mg Prot.) | 17.33 ± 1.58 | 871.66** ± 148.84<br>(+ 4930%) | 18.82 ± 3.30 | 554.00** ± 119.35<br>(+ 2844%) |
| UDPGT<br>(nmol/min/ mg Prot.) | 16.24 ± 0.56 | 17.23 ± 0.82<br>N.S. | 15.18 ± 1.28 | 12.96 ± 2.14<br>N.S. |

*: p≤0.05; **: p≤0.01

## III. CONCLUSION

These data indicate that phenobarbital has the ability in male C57BL/6J mice to:
- modify the thyroid hormone balance by causing a decrease in T4 and/or T3 levels and a concomitant increase in TSH,
- induce liver changes including in particular induction of several hepatic enzymes as total cytochrome P-450, PROD, BROD activities.

| | |
|---|---|
| **Report:** | **KIIA 5.5.4 /19; [redacted] 2008; M-308369-01** |
| Title: | AE C656948 Mechanistic 3-day toxicity study in the male mouse (pharmacokinetic investigations of the clearance of intravenously administered 125I-thyroxine) |
| Report No & Document No | SA 08159<br>M-308369-01 |
| **Guidelines:** | **No specific guideline** |
| **Deviations:** | **none** |
| **GLP** | **no** |

**Executive 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. On Study Day 4 each animal received by intravenous injection via the tail 250 µl 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 µl 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 both subgroups of animals 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 following $^{125}$I administration, 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, the 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 following $^{125}$I administration. In addition, the response observed with AE C656948 treated animals was similar to that noted in animals treated with 80 mg/kg/day Phenobarbital.

# I. MATERIALS AND METHODS

## A. MATERIALS:

**1. Test Material:** AE C656948
**Description:** Light beige powder
**Lot/Batch:** Mix-Batch:08528/0002
**Purity:** 94.7 %
**CAS:** 658066-35-4
**Stability of test compound:** Stable in rodent diet for a period covering the study duration

**2. Vehicle and /or positive control:** Vehicle for AE C656948: rodent diet
Positive control: Phenobarbital
Vehicle for Phenobarbital: methylcellulose 400

**Positive control:** Phenobarbital
**Description:** White crystalline powder
**Lot/Batch:** 06100228
**Purity:** 99.6 %
**CAS:** 50-06-6
**Stability of test compound:** Stable for a period covering the study duration

**3. Test animals:**
**Species**: Mouse – Male only
**Strain:** C57BL/6J
**Age:** 8 weeks approximately at start of treatment
**Weight at dosing:** 19.8 to 25.4 g
**Acclimation period:** 6 days (subgroup 1 animals), 23 days (subgroup 2 animals)
**Diet:** Certified rodent powdered / pelleted and irradiated diet A04C-10 P1 from [redacted] ([redacted], France), *ad libitum*
**Water:** Tap water, *ad libitum*
**Housing:** Animals were caged individually in suspended stainless steel wire mesh cages.
**Environmental conditions –**
**Temperature:** 20 – 24°C
**Humidity:** 40 – 70 %
**Air changes:** Approximately 10-15 changes per hour
**Photoperiod:** Alternating 12-hour light and dark cycles (7 am- 7 pm)

**B. STUDY DESIGN:**

**1. In life dates:** Start of treatment to final sacrifice from August 23 to 27, 2008, for the subgroup 1 animals and from September 05 to 09, 2008, for the subgroup 2 animals.

**2. Animal assignment and treatment**

The dose level for AE C656948 was set after evaluation of the results of a 28-day toxicity study (see section 5.3.1) and a 90-day toxicity study (see section 5.3.2) in the mouse with this substance. In the 28-day study, 5000 ppm clearly exceeded the maximum tolerated dose as all males and most females had to be sacrificed for humane reasons before the end of the study. In the 90-day study slight toxicity was seen at the highest dose tested, 1000 ppm. Therefore, the dose level of 2000 ppm was selected as an appropriate high dose level to be used in this study. Although, 2000 ppm was higher than the high dose used in the mouse carcinogenicity study (750 ppm), this dose level was selected to allow a clearer evaluation of the effects of AE C656948 without causing excessive toxicity. Males only were used in this study as an increased incidence of thyroid follicular cell adenoma was observed in this sex only. In addition, 2000 ppm AE C656948 was the dose level selected on other
*in-vivo* mechanistic studies in the mouse. Phenobarbital was administered once daily for 3 days by oral gavage to a group of 5 males at a dose level of 80 mg/kg/day in 0.5% aqueous solution of methycellulose 400. The dose level was selected on the basis of previous studies conducted with phenobarbital and was expected to provoke a positive response. A negative control group received untreated diet alone during the study period.
Animal housing and husbandry were in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals (Public Health Service, National Institute of Health, NIH publication N°86-23, revised 1985) and "Le Guide du Journal Officiel des Communautés Européennes L358, 18 Décembre 1986, N°86/609/CEE du 24 Novembre 1986".

**Table 5.5.4-62: Study design**

| Test group | Treatment | Dose level | Duration of treatment | Animals assigned |
|---|---|---|---|---|
| 1* | Control diet | 0 | 3 days | 5 |
| 2* | AE C656948 | 2000 ppm | 3 days | 5 |
| 3* | Phenobarbital | 80 mg/kg/day | 3 days | 5 |
| 4** | Control diet | 0 | 3 days | 1 |
| 5** | AE C656948 | 2000 ppm | 3 days | 4 |

* subgroup 1 animals ** subgroup 2 animals

The stability of AE C656948 in the diet at 20 and 10000 ppm has been demonstrated in a previous study after a freezing period of 95 days followed by storage periods at room temperature of 10 days or 105 days. Before the start of the study homogeneity and concentration were checked on the study mix at 2000 ppm. The mean value obtained from the homogeneity check was taken as measured concentration. The study mix was found to be acceptable for use on the study as the homogeneity and concentration checks were within the range of 91 to 93% of the nominal concentration. The stability of Phenobarbital in aqueous methylcellulose at 8 g/l has been demonstrated in a previous study after a storage period of 29 days. Before the start of the study homogeneity and concentration were checked on the study formulation at 8 g/l. The mean value obtained from the homogeneity check was taken as measured concentration. The study formulation was found to be acceptable for use on the study as the homogeneity and concentration checks were 100% of the nominal concentration.

**3. Diet and water**

Certified rodent powdered and irradiated diet A04CP1-10 from [redacted] ([redacted], France) and filtered and softened tap water from the municipal water supply in individual bottles were available *ad libitum* until Day 4. On transfer to the radioactive suite on Day 4, animals were given certified rodent pelleted and irradiated diet A04CP1-10 from [redacted] ([redacted], France). The water was supplied via water bottles. Animals were not diet fasted overnight prior to blood sampling and final sacrifice. Routine analyses of food and water indicated that there was no contamination which could have compromised the study.

**4. Statistics**

Body weight
Mean and standard deviation were calculated for each group.
The F test was performed to compare the homogeneity of group variances.
If the F test was not significant ($p>0.05$), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided).
If the F test was significant ($p\leq0.05$), data were transformed using the log transformation. If the F test on log transformed data was not significant ($p>0.05$), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided) on log transformed data.
If the F test was significant ($p\leq0.05$) even after log transformation, the mean of the exposed group was compared to the mean of the control group using the modified t-test (2-sided).
If one or more group variance(s) equal 0, means were compared using non-parametric Mann-Whitney test (2-sided).
Group means will be compared at the 5% and 1% levels of significance.
Statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics).

**C. METHODS:**

**1. Observations**

The animals were observed twice daily for moribundity and mortality (once daily on weekends or public holidays). Observed clinical signs were recorded at least once daily for all animals and detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility, and duration of any clinical signs were recorded.

**2. Body weight**

Each animal was weighed at least once during the acclimatization phase, then on Study Days 1 and 4.

**3. Food consumption**

Food consumption was not recorded.

**4. Intravenous injection of $^{125}$I-Thyroxine**

$^{125}$I-Thyroxine solution ([redacted], France) was diluted with 0.9 % sterile saline solution to provide a solution containing 4 µCi/ml. The certificate of analysis of the stock solution of $^{125}$I-Thyroxine is presented in Attachment 2 in the study report. Each animal received on Day 4 by intravenous injection via the tail 250 µl of the diluted $^{125}$I-Thyroxine solution corresponding to 1 µCi of $^{125}$I-Thyroxine per animal. Approximately 3 hours after the intravenous injection of

$^{125}$I-Thyroxine, each animal received 0.1 mg of NaI in 250 µl of 0.9 % sterile saline by intraperitoneal injection. Dilution of the $^{125}$I-Thyroxine solution and preparation of the NaI solution was carried out on the day of administration.

For the animals belonging to subgroup 2, to take into account the decay of the specific activity of the $^{125}$I-Thyroxine, a new dilution of the $^{125}$I-Thyroxine stock solution was performed. This diluted solution was performed by mixing the same volumes of stock solution of $^{125}$I-Thyroxine and 0.9 % sterile saline as the ones used for the main experiment (subgroup 1 animals).

**5. Blood sampling:**

Animals were not diet fasted prior to blood collection.

A whole blood sample was collected from the retro-orbital venous plexus of each surviving animal. Whole blood samples were placed into tubes and the radioactivity were measured. Prior to blood sampling animals were anesthetized with Isoflurane ([redacted], France). Samples were taken at 1 hour 20 min, 2, 4, 6, and 24 hours after the dosing with the $^{125}$I-Thyroxine.

**6. Measurement of radioactivity:**

Two aliquots of 50 µl of each whole-blood sample were dispensed directly into tubes for radioactivity measurement. The radioativity ($^{125}$I) was measured at the same time in all the blood samples generated both in subgroup 1 and subgroup 2 animals.

Radioativity ($^{125}$I) were measured using a Cobra gamma scintillation counter (Packard).

Radioactivity measurement was expressed as follows:

Whole blood radioactive content.

Data from animals belonging to both subgroups 1 and 2 were combined and an overall mean and standard deviation derived for each treatment group.

## II. RESULTS AND DISCUSSION

### A. OBSERVATIONS:

Mortality:

There was no mortality in any groups throughout the study.

Clinical signs:

There were no clinical signs observed.

### B. BODY WEIGHT:

After 3-days of exposure, no statistically significant effect on mean body weight was recorded either in the AE C656948 or Phenobarbital treated animals when compared to the controls.

## C. T4 CLEARANCE:

During the course of the study, some technical difficulties affected the process of intravenous injection of the initial batch of animals allocated to the study. Consequently, Thyroxine (T4) blood level data was obtained from 9 animals only (5 control animals, 1 AE C656948 treated animal and 3 Phenobarbital treated animals). Consequently, 5 additional animals were incorporated onto the study which consisted of 1 control animal and 4 AE C656948 treated animals. T4 blood level data was obtained from these 5 animals. The data presented in the table below represent the combined mean and standard deviation derived from both subgroups of animals for each treatment level.

**Table 5.5.4-63: Whole blood radioactivity after a single administration of $^{125}$I-Thyroxine Mean ± Standard deviation (cpm)**

| | Control | AE C656948 (2000 ppm) | Phenobarbital (80 mg/kg/day) |
|---|---|---|---|
| Number of animals | 6 | 5 | 3 |
| Time point 1h20min | 11434 ± 1624 | 4767 ±1953 | 5775 ±2615 |
| Time point 2h | 11025 ±1415 | 4686 ±1999 | 5905 ±2095 |
| Time point 4h | 9811±1756 | 4984 ±1491 | 5651 ± 995 |
| Time point 6h | 8692 ±1397 | 4566 ± 1342 | 6021 ±1046 |
| Time point 24h | 2686 ± 454 | 1955 ± 199 | 2309 ± 446 |

The results show after an intravenous injection of $^{125}$I-Thyroxine a decrease of the radioactivity in the blood of the AE C656948 treated animals when compared to 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 following $^{125}$I administration, compared with the controls.

Similarly, the decrease of the radioactivity was also observed in animals treated with Phenobarbital a reference compound known to induce an increase in Thyroxine clearance in the mouse through induction of Thyroxine glucoronidation.

Overall these results indicate that the Thyroxine clearance was increased in the AE C656948 treated animals when compared to control animals.

## III. CONCLUSION

In conclusion, the 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 following $^{125}I$ administration. In addition, the response observed with AE C656948 treated animals was similar to that noted in animals treated with 80 mg/kg/day Phenobarbital.

| | |
|---|---|
| **Report:** | KIIA 5.5.4 /20; [redacted] 2008; M-308073-01 |
| Title: | AE C656948 Mechanistic 3-day toxicity study in the male mouse (qPCR investigations of gene transcripts in the liver) |
| Report No & | SA 08151 |
| Document No | M-308073-01 |
| **Guidelines:** | **No specific guideline** |
| **Deviations:** | **None** |
| **GLP** | **No** |

**Executive 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%, $p \leq 0.01$) and UDP glucoronosyltransferase transcripts (from +173% to +273%, $p \leq 0.01$). Similarly, an up-regulation of sulfotransferase transcripts Sult 1a1 and Sultn (+62% and +96%, respectively, $p \leq 0.01$) and UDP glucoronosyltransferase transcripts (from +82% to +119%, $p \leq 0.01$) 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.

# I. MATERIALS AND METHODS

## A. MATERIALS:

**1. Test Material:** AE C656948
**Description:** Light beige powder
**Lot/Batch:** Mix-Batch: 08528/0002
**Purity:** 94.7 %
**CAS:** 658066-35-4
**Stability of test compound:** Stable in rodent diet for a period covering the study duration

**2. Vehicle and /or positive control:** Vehicle for AE C656948: rodent diet
Positive control: Phenobarbital
Vehicle for Phenobarbital: methylcellulose 400

**Positive control:** Phenobarbital
**Description:** White crystalline powder
**Lot/Batch:** 06100228
**Purity:** 99.6 %
**CAS:** 50-06-6
**Stability of test compound:** Stable for a period covering the study duration

**3. Test animals:**
**Species**: Mouse – Male only
**Strain:** C57BL/6J
**Age:** 8 weeks approximately at start of treatment
**Weight at dosing:** 19.9 to 23.4 g
**Acclimation period:** 6 days
**Diet:** Certified rodent powdered and irradiated diet A04C-10 P1 from [redacted] ([redacted], [redacted], France), *ad libitum*
**Water:** Tap water, *ad libitum*
**Housing:** Animals were caged individually in suspended stainless steel wire mesh cages.
**Environmental conditions –**
**Temperature:** 20 – 24°C
**Humidity:** 40 – 70 %
**Air changes:** Approximately 10-15 changes per hour
**Photoperiod:** Alternating 12-hour light and dark cycles (7 am- 7 pm)

## B. STUDY DESIGN:

**1. In life dates:** Start of treatment to final sacrifice from July 08 to 11, 2008.

**2. Animal assignment and treatment**

The dose level for AE C656948 was set after evaluation of the results of a 28-day toxicity study (see section 5.3.1) and a 90-day toxicity study (see section 5.3.2) in the mouse with this substance. In the 28-day study, 5000 ppm clearly exceeded the maximum tolerated dose as all males and most females had to be sacrificed for humane reasons before the end of the study. In the 90-day study slight toxicity was seen at the highest dose tested, 1000 ppm. Therefore, a dose level of 2000 ppm was selected as an appropriate high dose level to be used in this study. Although, 2000 ppm was higher than the high dose used in the mouse carcinogenicity study (750 ppm), this dose level was selected to allow a clearer evaluation of the effects of AE C656948 without causing excessive toxicity. Males only were used in this study as an increased incidence of thyroid follicular cell adenoma was observed in this sex only. In addition, 2000 ppm AE C656948 was the dose level selected on other
*in-vivo* mechanistic studies in the mouse. Phenobarbital was administered once daily for 3 days by oral gavage to a group of 5 males at a dose level of 80 mg/kg/day in 0.5% aqueous solution of methycellulose 400. The dose level was selected on the basis of previous studies conducted with phenobarbital and was expected to provoke a positive response. A negative control group received untreated diet alone during the study period.
Animal housing and husbandry were in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals (Public Health Service, National Institute of Health, NIH publication N°86-23, revised 1985) and "Le Guide du Journal Officiel des Communautés Européennes L358, 18 Décembre 1986, N°86/609/CEE du 24 Novembre 1986".

**Table 5.5.4-64: Study design**

| Test group | Treatment | Dose level | Duration of treament | Animals assigned |
|---|---|---|---|---|
| 1 | Control diet | 0 | 3 days | 10 |
| 2 | AE C656948 | 2000 ppm | 3 days | 10 |
| 3 | Phenobarbital | 80 mg/kg/day | 3 days | 10 |

The stability of AE C656948 in the diet at 20 and 10000 ppm has been demonstrated in a previous study after a freezing period of 95 days followed by storage periods at room temperature of 10 days or 105 days. Before the start of the concentration were checked on the study mix at 2000 ppm. The study mix was found to be acceptable for use on the study as the concentration check was 99% of the nominal concentration. The stability of Phenobarbital in aqueous methylcellulose at 8 g/l has been demonstrated in a previous study after a storage period of 29 days. Before the start of the study concentration was checked on the study formulation at 8 g/l. The study formulation was found to be acceptable for use on the study as the concentration check was 100% of the nominal concentration.

**3. Diet and water**

Certified rodent powdered and irradiated diet A04CP1-10 from [redacted] ([redacted], France) and filtered and softened tap water from the municipal water supply in individual bottles were available *ad libitum*. Animals were not diet fasted overnight prior final sacrifice. Routine analyses of food and water indicated that there was no contamination which could have compromised the study.

**4. Statistics**

- Body weight parameters
- Terminal body weight, absolute and relative organ weights parameters
- Organ weight parameters
- qPCR data

Mean and standard deviation were calculated for each group.

• Terminal body weight, absolute and relative organ weight parameters.
The F test was performed to compare the homogeneity of group variances.
If the F test was not significant ($p>0.05$), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided).
If the F test was significant ($p\leq0.05$), mean of the exposed group was compared to the mean of the control group using the modified t-test (2-sided).

• Body weight
The F test was performed to compare the homogeneity of group variances.
If the F test was not significant ($p>0.05$), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided).
If the F test was significant ($p\leq0.05$), data were transformed using the log transformation. If the F test on log transformed data was not significant ($p>0.05$), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided) on log transformed data.
If the F test was significant ($p\leq0.05$) even after log transformation, mean of the exposed group was compared to the mean of the control group using the modified
t-test (2-sided).

If one or more group variance(s) equaled 0, means were compared using the non parametric Mann-Whitney test (2-sided).

• qPCR data
The objective of the statistical analysis was to compare mean quantities of 9 gene transcripts calculated in the AE C656948 treated group to the mean quantities of the 9 gene transcripts calculated in the control group. Mean quantities of the 9 gene transcripts calculated in the Phenobarbital treated group were also compared to the mean quantities calculated in the control group.

Because of a lack of normalization and a high variability between calculated values of gene transcript quantities, non-parametric procedures were selected. The mean quantity of each gene transcript calculated in the AE C656948 treated group was compared to the mean quantity calculated in the control group using the exact Mann-Whitney test (2-sided). An exact non-parametric test was preferred because of the low sample sizes (10 animals per group).

Group means were compared at the 5% and 1% levels of significance.
Statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics) except for qPCR data which were analyzed using SAS programs.

## C. METHODS:

### 1. Observations

The animals were observed twice daily for moribundity and mortality (once daily on weekends or public holidays). Observed clinical signs were recorded at least once daily for all animals and detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility, and duration of any clinical signs were recorded.

### 2. Body weight

Each animal was weighed at least once during the acclimatization phase, then on Study Days 1 and 4.

### 3. Food consumption

Food consumption was not recorded.

### 4. Necropsy procedure and tissue collection

On Study Day 4 all animals were sacrificed. All sacrifices were performed by exsanguination under deep anesthesia (inhalation of Isoflurane; [redacted], France). Animals were not diet fasted overnight prior to sacrifice. All animals were necropsied. The necropsy included the examination of all major organs, tissues and body cavities. Macroscopic abnormalities were recorded but not sampled except upon decision of the Study Pathologist.
The liver was weighed fresh at scheduled sacrifice.
A piece of median and left lobe liver of all the animals was collected and stored frozen at approximately -70°C for quantitative Polymerase Chain Reaction analysis. The remaining portions of livers were discarded.

### 5. qPCR analysis:

#### Total RNA purification

Total cytoplasmic RNA was isolated from the liver of individual control and treated animals using RNeasy Midi kits (Qiagen). RNA quality controls were performed based on the ribosomal RNA electrophoretic profiles using a Bioanalyser (Agilent Technologies).

#### Quantitative PCR

Ten µg of total RNA was used for Reverse transcription (RT) using a High Capacity cDNA Archive kit (Applied Biosystems). The assay was performed in duplicate using Taqman assays (Assay on demand, Applied Biosystems), 1/50 diluted first strand cDNA, AmpliTaq Gold® PCR Master Mix on an ABI prism 7900 HT machine (Applied Biosystems). For each gene transcript measured, a negative control condition was included in which H2O MQ was used as template instead of first strand cDNA.

**Table 5.5.4-65: List of Taqman assays used**

| Gene family | Isoform | Refset ID | Taqman assay ID (Applied Biosystems) |
|---|---|---|---|
| Cytochrome P450 | Cyp1a1 | NM_009992.3 | Mm00487218_m1 |
| Cytochrome P450 | Cyp2b9 | NM_010000.2 | Mm00657910_m1 |
| Cytochrome P450 | Cyp3a11 | NM_007818.3 | Mm00731567_m1 |
| Sulfotransferase | Sulta1 | NM_133670.1 | Mm00467072_m1 |
| Sulfotransferase | Sulta2 | NM_009286.1 | Mm02394381_g1 |
| Sulfotransferase | Sultn | NM_016771.2 | Mm00502030_m1 |
| UDP glucuronosyltransferase | Ugt1a1 | NM_201645.2 | Mm02603337_m1 |
| UDP glucuronosyltransferase | Ugt2b1 | NM_152811.1 | Mm00514184_m1 |
| UDP glucuronosyltransferase | Ugt2b5 | NM_009467.1 | Mm01623253_s1 |
| Beta-2 microglobulin | B2m | NM_009735.3 | Mm00437762_m1 |

Beta-2 microglobulin (B2m) was selected as reference gene for the quantitative calculations of transcripts. The relative quantity (RQ) value of each test transcript was calculated using the following formula:

$\Delta\Delta Ct = (Ct_{test} - Ct_{B2m})_{treated} - (Ct_{test} - Ct_{B2m})_{control}$

$RQ = 2^{-\Delta\Delta Ct}$

where Ct is the threshold cycle at which PCR amplification started to be significantly different from the background signal.

Each RQ value obtained for a given gene was normalized by dividing by the RQ value obtained for the control animal ST1M2625.

## II. RESULTS AND DISCUSSION

### A. OBSERVATIONS:

Mortality:

There was no mortality in any groups throughout the study.

Clinical signs:

Reduced motor activity was recorded throughout the treatment period in the Phenobarbital treated animals. No other clinical signs were observed during the course of study in any animals.

### B. BODY WEIGHT:

After 3-days of exposure, no effect on mean body weight was recorded either in the AE C656948 or Phenobarbital treated animals when compared to the controls.

### C. SACRIFICE AND QPCR ANALYSIS

Mean liver and mean liver weight to body weight ratio were statistically significantly higher both in AE C656948 and Phenobarbital treated animals when compared to control animals.

**Table 5.5.4-66: Liver weight changes (% change when compared to controls)**

| Sex | Males | | |
|---|---|---|---|
| Test substance | Control Diet | AE C656948 (2000 ppm) | Phenobarbital (80 mg/kg/day) |
| Mean absolute liver weight (g) | 1.20±0.10 | 1.93**±0.12 (+61%) | 1.40**±0.13 (+17%) |
| Mean liver to body weight ratio (%) | 5.34±0.36 | 8.55**±0.40 (+60%) | 6.34**±0.29 (+19%) |

**** p≤0.01**

**QPCR ANALYSIS**

**Table 5.5.4-67: Mean Relative Quantity ± standard deviation of gene transcripts (% change compared to control mean values)**

| Gene transcripts | Control | AE C656948 (2000 ppm) | Phenobarbital (80 mg/kg/day) |
|---|---|---|---|
| Cyp1a1 | 1.29 ± 0.30 | 4.81** ± 1.19 (+272%) | 1.20 ± 0.24 NS (-7%) |
| Cyp2b9 | 14.71 ± 30.17 | 48.61* ± 91.21 (+230%) | 21.11 ± 30.19 NS (+43%) |
| Cyp3a11 | 1.51 ± 0.56 | 43.59** ± 34.38 (+2783%) | 7.76** ± 4.11 (+413%) |
| Sult1a1 | 1.19 ± 0.39 | 2.29** ± 1.20 (+92%) | 1.92* ± 0.46 (+62%) |
| Sult2a2 | 0.51 ± 0.22 | 2.90** ± 2.08 (+463%) | 0.63 ± 0.35 NS (+22%) |
| Sultn | 1.41 ± 0.56 | 5.93**± 3.90 (+321%) | 2.76** ± 1.18 (+96%) |
| Ugt1a1 | 1.08 ± 0.16 | 4.03** ± 0.80 (+273%) | 2.36** ± 0.26 (+119%) |
| Ugt2b1 | 1.04 ± 0.25 | 2.84** ± 0.71 (+173%) | 1.97** ± 0.53 (+90%) |
| Ugt2b5 | 1.30 ± 0.23 | 4.30** ± 2.16 (+231%) | 2.36** ± 0.87 (+82%) |

NS : Not statistically significant
* : Statistically different from the control group (p≤0.05)
** : Statistically different from the control group (p≤0.01)

Cyp1a, Cyp2b and Cyp3a gene transcript isoforms were up-regulated in the liver of AE C656948 treated animals when compared with controls. In the Phenobarbital treated animals the induction of Cyp3a was statistically significantly up-regulated (+413%, p≤0.01). The Cyp2b transcript was only slightly up-regulated (+43%) and the effect was not statistically significant.
The transcripts of isoforms of sulfotransferase and UDP glucoronosyltransferase known to inactivate T3 and T4 via glucoronide and sulfate derivatives were statistically significantly up-regulated in the liver of AE C656948 treated animals. In the liver of animals treated with Phenobarbital, except for Sult2a2, the same Sult and Ugt transcripts were statistically significantly up-regulated as with AE C656948 treated animals.

## III. CONCLUSION

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.

| | |
|---|---|
| **Report:** | **KIIA 5.5.4 /21; [redacted] 2009; M-328662-01** |
| Title: | AE C656948 Definitive Mechanistic 4-day toxicity study in the male mouse (pharmacokinetic investigations of the clearance of intravenously administered 125I-thyroxine) |
| Report No & Document No | SA 08288<br>M-328662-01 |
| **Guidelines:** | **No specific guideline** |
| **Deviations:** | **none** |
| **GLP** | **yes** |

**Executive 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 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 glucoronidation and sulfonation. On Study Day 5 each animal received by intravenous injection via the tail 250 µl of diluted $^{125}$I-Thyroxine solution in 0.9% sterile saline. Approximately 3 hours post-administration with $^{125}$I-Thyroxine, each animal received 0.1 mg of NaI in 250 µl of 0.9% sterile saline by intraperitoneal injection. A blood sample was collected from the retro-orbital venous plexus of each animal after 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 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 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 procedure. 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 following $^{125}$I administration, 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, the 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 following $^{125}$I administration. In addition, the response observed with AE C656948 treated animals was similar to that noted in animals treated with 80 mg/kg/day Phenobarbital.

# I. MATERIALS AND METHODS

## A. MATERIALS:

**1. Test Material:** AE C656948
**Description:** Light beige powder
**Lot/Batch:** Mix-Batch:08528/0002
**Purity:** 94.7 %
**CAS:** 658066-35-4
**Stability of test compound:** Stable in rodent diet for a period covering the study duration

**2. Vehicle and /or positive control:** Vehicle for AE C656948: rodent diet
Positive control: Phenobarbital
Vehicle for Phenobarbital: methylcellulose 400

**Positive control:** Phenobarbital
**Description:** White crystalline powder
**Lot/Batch:** 06100228
**Purity:** 99.6 %
**CAS:** 50-06-6
**Stability of test compound:** Stable for a period covering the study duration

**3. Test animals:**
**Species**: Mouse – Male only
**Strain:** C57BL/6J
**Age:** 8 weeks approximately at start of treatment
**Weight at dosing:** 20.3 to 23.5 g
**Acclimation period:** at least 9 days
**Diet:** Certified rodent powdered / pelleted and irradiated diet A04C-10 P1 from [redacted] ([redacted], France), *ad libitum*
**Water:** Tap water, *ad libitum*
**Housing:** Animals were caged individually in suspended stainless steel wire mesh cages.
**Environmental conditions –**
**Temperature:** 20 – 24°C
**Humidity:** 40 – 70 %
**Air changes:** Approximately 10-15 changes per hour
**Photoperiod:** Alternating 12-hour light and dark cycles (7 am- 7 pm)

**B. STUDY DESIGN:**

**1. In life dates:** Start of treatment to final sacrifice from Novemver 14 to 19, 2008, for the subgroup 1 animals and from November 16 to 21, 2008, for the subgroup 2 animals.

**2. Animal assignment and treatment**

The dose level for AE C656948 was set after evaluation of the results of a 28-day toxicity study (see section 5.3.1) and a 90-day toxicity study (see section 5.3.2) in the mouse with this substance. In the 28-day study, 5000 ppm clearly exceeded the maximum tolerated dose as all males and most females had to be sacrificed for humane reasons before the end of the study. In the 90-day study slight toxicity was seen at the highest dose tested, 1000 ppm. Therefore, the dose level of 2000 ppm was selected as an appropriate high dose level to be used in this study. Although, 2000 ppm was higher than the high dose used in the mouse carcinogenicity study (750 ppm), this dose level was selected to allow a clearer evaluation of the effects of AE C656948 without causing excessive toxicity. Males only were used in this study as an increased incidence of thyroid follicular cell adenoma was observed in this sex only. In addition, 2000 ppm AE C656948 was the dose level selected on other *in-vivo* mechanistic studies in the mouse. Phenobarbital was administered once daily for 4 days by oral gavage to a group of 8 males at a dose level of 80 mg/kg/day in 0.5% aqueous solution of methycellulose 400. The dose level was selected on the basis of previous studies conducted with phenobarbital and was expected to provoke a positive response. A negative control group received untreated diet alone during the study period.
Animal housing and husbandry were in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals (Public Health Service, National Institute of Health, NIH publication N°86-23, revised 1985) and "Le Guide du Journal Officiel des Communautés Européennes L358, 18 Décembre 1986, N°86/609/CEE du 24 Novembre 1986".

**Table 5.5.4-68: Study design**

| Test group | Treatment | Dose level | Duration of treatment | Animals assigned |
|---|---|---|---|---|
| 1 | Control diet | 0 | 4 days | 8 |
| 2 | AE C656948 | 2000 ppm | 4 days | 8 |
| 3 | Phenobarbital | 80 mg/kg/day | 4 days | 8 |

The stability of AE C656948 in the diet at 20 and 10000 ppm has been demonstrated in a previous study after a freezing period of 95 days followed by storage periods at room temperature of 10 days or 105 days. Before the start of the study homogeneity and concentration were checked on the study mix at 2000 ppm. The mean value obtained from the homogeneity check was taken as measured concentration. The study mix was found to be acceptable for use on the study as the homogeneity and concentration checks were within the range of 97 to 98% of the nominal concentration. The stability of Phenobarbital in aqueous methylcellulose at 8 g/l has been demonstrated in a previous study after a storage period of 29 days. Before the start of the study homogeneity and concentration were checked on the study formulation at 8 g/l. The mean value obtained from the homogeneity check was taken as measured concentration. The study formulation was found to be acceptable for use on the study as the homogeneity and concentration checks were 99% of the nominal concentration.

**3. Diet and water**

Certified rodent powdered and irradiated diet A04CP1-10 from [redacted] ([redacted], France) and filtered and softened tap water from the municipal water supply in individual bottles were available *ad libitum* until Day 5. On transfer to the radioactive suite on Day 5, animals were given certified rodent pelleted and irradiated diet A04C-10 from [redacted] ([redacted], France). The water was supplied via water bottles. Animals

were not diet fasted overnight prior to blood sampling and final sacrifice. Routine analyses of food and water indicated that there was no contamination which could have compromised the study.

**4. Statistics**

Body weight
Mean and standard deviation were calculated for each group.
The F test was performed to compare the homogeneity of group variances.
If the F test was not significant ($p>0.05$), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided).
If the F test was significant ($p\leq0.05$), data were transformed using the log transformation. If the F test on log transformed data was not significant ($p>0.05$), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided) on log transformed data.
If the F test was significant ($p\leq0.05$) even after log transformation, the mean of the exposed group was compared to the mean of the control group using the modified t-test (2-sided).
If one or more group variance(s) equal 0, means were compared using non-parametric Mann-Whitney test (2-sided).
Group means will be compared at the 5% and 1% levels of significance.
Statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics).

Radioactivity measurements in the blood

For each animal, 2 replicated values were measured. Statistical analyses were conducted on the averaged values calculated per animal from the 2 replicate values.

Comparison between the subgroup 1 and subgroup 2
In a first step, mean of the subgroup 1 was compared to the mean of the subgroup 2 per time point and for each group by the use of the t-test when the subgroup variances were homogeneous or by the use of the modified t-test when the subgroup variances were not homogeneous. Tests performed were 2-sided.

Results indicated that for each group, the mean of the subgroup 1 was not statistically different from the mean of the subgroup 2 per time point. Consequently animals of the 2 subgroups were pooled for further analysis.

Comparison between the control and the AE C656948 treated group
Statistical comparisons were performed per time point and on pooled data of subgroups 1 and 2.
Group variances were compared by the use of the F test (2-sided).
When the F test was not significant ($p>0.05$), means were compared using the t-test (1-sided).
When the F test was significant ($p\leq0.05$), means were compared using the modified t-test (1-sided).

Comparison between the control and the Phenobarbital treated group
Statistical comparisons were performed per time point and on pooled data of subgroups 1 and 2.
Group variances were compared by the use of the F test (2-sided).
When the F test was not significant ($p>0.05$), means were compared using the t-test (1-sided).
When the F test was significant ($p\leq0.05$), means were compared using the modified t-test (1-sided).

Group means were compared at the 5% and 1% levels of significance.
Statistical analyses were carried out using SAS programs (Version 8.2).

## C. METHODS:

### 1. Observations

The animals were observed twice daily for moribundity and mortality (once daily on weekends or public holidays). Observed clinical signs were recorded at least once daily for all animals and detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility, and duration of any clinical signs were recorded.

### 2. Body weight

Each animal was weighed at least once during the acclimatization phase, then on Study Days 1 and 5.

### 3. Food consumption

Food consumption was not recorded.

### 4. Intravenous injection of $^{125}$I-Thyroxine

$^{125}$I-Thyroxine solution ([redacted], France) was diluted with 0.9 % sterile saline solution to provide a solution containing 4 µCi/ml. The certificate of analysis of the stock solution of $^{125}$I-Thyroxine is presented in Attachment 2 in the study report. Each animal received on Day 5 by intravenous injection via the tail 250 µl of the diluted $^{125}$I-Thyroxine solution corresponding to 1 µCi of $^{125}$I-Thyroxine per animal. Approximately 3 hours after the intravenous injection of $^{125}$I-Thyroxine, each animal received 0.1 mg of NaI in 250 µl of 0.9 % sterile saline by intraperitoneal injection. Dilution of the $^{125}$I-Thyroxine solution and preparation of the NaI solution was carried out on the day of administration.

### 5. Blood sampling:

Animals were not diet fasted prior to blood collection.

A whole blood sample was collected from the retro-orbital venous plexus of each surviving animal. Whole blood samples were placed into tubes and the radioactivity were measured. Prior to blood sampling animals were anesthetized with Isoflurane ([redacted], France). Samples were taken at 40 minutes, 1.5, 4 and 24 hours after the dosing with the $^{125}$I-Thyroxine.

### 6. Measurement of radioactivity:

Two aliquots of 50 µl of each whole-blood sample were dispensed directly into tubes for radioactivity measurement. The radioativity ($^{125}$I) was measured at the same time in all the blood samples generated both in subgroup 1 and subgroup 2 animals.

Radioativity ($^{125}$I) were measured using a Cobra gamma scintillation counter (Packard).

Radioactivity measurement was expressed as follows:

Whole blood radioactive content.

Data from animals belonging to both subgroups 1 and 2 were combined and an overall mean and standard deviation derived for each treatment group.

## II. RESULTS AND DISCUSSION

### A. OBSERVATIONS:

Mortality:

There was no mortality in any group during the 4 day treatment period. On Day 5, one animal from the Phenobarbital treated group was sacrificed due to a technical problem with the intravenous injection of the $^{125}$I-Thyroxine solution.

Clinical signs:

There were no clinical signs observed.

### B. BODY WEIGHT:

After 4 days of exposure, no statistically significant effect on mean body weight was recorded either in the AE C656948 or Phenobarbital treated animals when compared to the controls.

### C. T4 CLEARANCE:

The data presented in the table below represent the combined mean and standard deviation derived from both subgroups of animals for each treatment level at each time point. It can be estimated; knowing that the circulating blood volume in a mouse is approximately 80 ml/kg, that at time point 0, the amount of radioactivity in 50 µl of blood of all the animals was approximately 40000 cpm.

**Table 5.5.4-69: Whole blood radioactivity after a single administration of $^{125}$I-Thyroxine Mean ± Standard deviation (cpm)**

| | Control | AE C656948 (2000 ppm) | Phenobarbital (80 mg/kg/day) |
|---|---|---|---|
| Number of animals | 8 | 8 | 7 |
| Time point 40 min | 19726 ± 1468 | 6163** ± 2025 | 10691** ± 1197 |
| Time point 1h 30 min | 16930 ± 1001 | 6388** ± 1982 | 10592** ± 1245 |
| Time point 4h | 13781 ± 1099 | 6111** ± 1304 | 9312** ± 1330 |
| Time point 24h | 3889 ± 561 | 2562 ** ± 482 | 2653** ± 547 |

** $p \leq 0.01$

The results show after an intravenous injection of $^{125}$I-Thyroxine there was a statistically significantly lower level of radioactivity present in the blood of animals treated with AE C656948, at all time points, when compared with the controls. This decrease is considered to reflect a more rapid clearance of Thyroxine in AE C656948 treated animals over a 24 hour period, when comparec with the controls.

Similarly, a decrease of the radioactivity level was also observed in animals treated with Phenobarbital, a reference compound known to induce an increase in Thyroxine clearance in the mouse through induction of Thyroxine glucoronidation.

Overall these results indicate that the Thyroxine clearance was increased in the AE C656948 treated animals when compared to control animals.

## III. CONCLUSION

In conclusion, the 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 following $^{125}I$ administration. In addition, the response observed with AE C656948 treated animals was similar to that noted in animals treated with 80 mg/kg/day Phenobarbital.

| | |
|---|---|
| **Report:** | **KIIA 5.5.4 /26; [redacted] 2011; M-408352-01** |
| Title: | Fluopyram: Mechanistic 3-day toxicity study in the mouse by oral gavage (thyroid hormone investigations) |
| Report No & Document No | SA 10241<br>M-408352-01 |
| **Guidelines:** | **No specific guideline** |
| **Deviations:** | **none** |
| **GLP** | **no** |

**Executive 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.

# I. MATERIALS AND METHODS

## A. MATERIALS:

**1. Test Material:** Fluopyram (AE C656948)
**Description:** Beige powder
**Lot/Batch:** Mix-Batch: 08528/0002
**Purity:** 94.7 %
**CAS:** 658066-35-4
**Stability of test compound:** Stable in 0.5% aqueous solution of methylcellulose for a period covering the study duration

**2. Vehicle and /or positive control:** Vehicle for AE C656948: methylcellulose
Phenobarbital (positive control)
Vehicle for Phenobarbital: methylcellulose
**Positive control:** Phenobarbital
**Description:** White crystalline powder
**Lot/Batch:** Lot No. 06100228
**Purity:** 99.6 %
**CAS:** 50-06-6
**Stability of test compound:** Stable in 0.5% aqueous solution of methylcellulose for a period covering the study duration

**3. Test animals:**
**Species**: Mouse – Male only
**Strain:** C57BL/6J
**Age:** 8 weeks approximately at start of treatment
**Weight at start of dosing:** 21.1 to 25.7 g
**Acclimation period:** 5 days
**Diet:** Certified rodent powdered and irradiated diet A04C-10 P1 from [redacted] ([redacted], [redacted], France), *ad libitum*
**Water:** Tap water, *ad libitum*
**Housing:** Animals were caged individually in suspended stainless steel wire mesh cages.
**Environmental conditions –**
**Temperature:** 20 – 24°C
**Humidity:** 40 – 70 %
**Air changes:** Approximately 10-15 changes per hour
**Photoperiod:** Alternating 12-hour light and dark cycles (7 am- 7 pm)

## B. STUDY DESIGN:

**1. In life dates:** Dosing period from September 27 to 30, 2010.

**2. Animal assignment and treatment**

The dose levels for Fluopyram were set after evaluation of the results from previous studies conducted with Fluopyram and following discussions with the US (EPA) and Canadian (PMRA) authorities. The dose level of 100 mg/kg/day is equivalent to the top dose level (750 ppm) used in the mouse cancer bioassay in which thyroid tumors were observed in the males (SA 05094) and the dose level of 300 mg/kg/day is equivalent to the dose level used in previous mouse mechanistic studies conducted with Fluopyram (SA 07215, SA 08151 and SA 08288), where thyroid hormone changes were detected.
The dose level of 80 mg/kg/day for Phenobarbital is the dose level used in previous mouse mechanistic studies conducted with Phenobarbital, where thyroid changes were detected
(SA 07326, SA 08151 and SA 08288).

**Table 5.5.4-70: Study design**

| GROUP | TEST SUBSTANCE | DOSE LEVEL (mg/kg/day) | NUMBER OF ANIMALS PER GROUP | ANIMAL IDENTITY | |
|---|---|---|---|---|---|
| MALES | | | | Subgroup 1 | Subgroup 2 |
| 1 | Control | 0 | 15 | UT1M4180 to 4186 | UT1M4187 to 4194 |
| 2 | Fluopyram | 100 | 15 | UT2M4195 to 4201 | UT2M4202 to 4209 |
| 3 | | 300 | 15 | UT3M4210 to 4216 | UT3M4217 to 4224 |
| 4 | Phenobarbital | 80 | 15 | UT4M4225 to 4231 | UT4M4232 to 4239 |

**3. Dosing preparation and analysis**

Fluopyram and Phenobarbital were suspended in 0.5% aqueous solution of methylcellulose to provide the required concentration. There was one preparation of the test formulation for the entire study. When not in use the formulations were stored at approximately +4°C.

The stability of Fluopyram at 10 g/l and 30 g/l has been demonstrated in a previous study (SA 04060), which covers the period of storage and usage for the current study.

The stability of Phenobarbital at 8 g/l has been demonstrated in a previous study (SA 03310), which covers the period of storage and usage for the current study.

**4. Statistics**

Variables analyzed

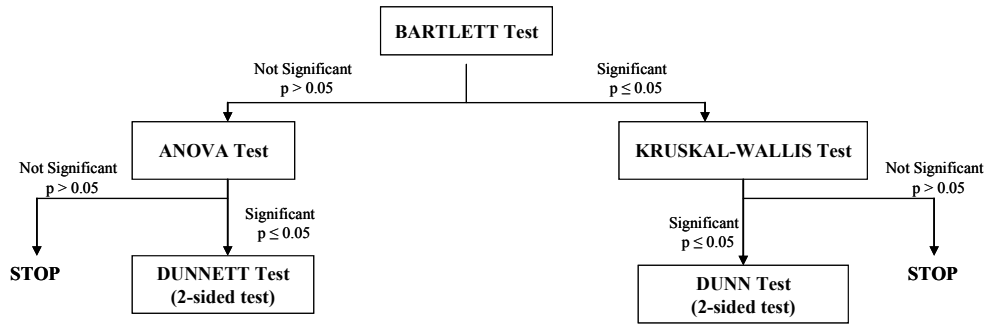
- Body weight parameters
- Hormonal parameters in the plasma
- Hormonal parameters in the bile

Statistical methods

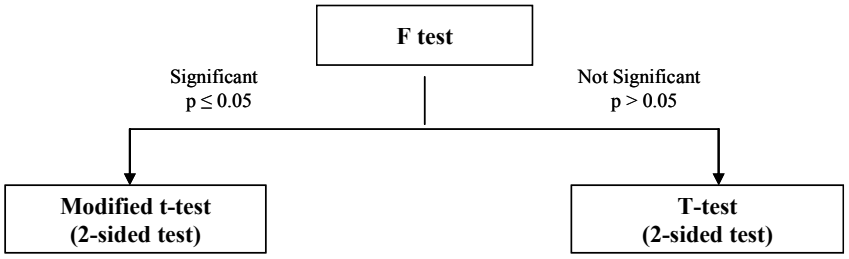
Mean and standard deviation were calculated for each group.

– Body weight change parameters,

**Fluopyram: comparison between the treated groups and the vehicle control group**



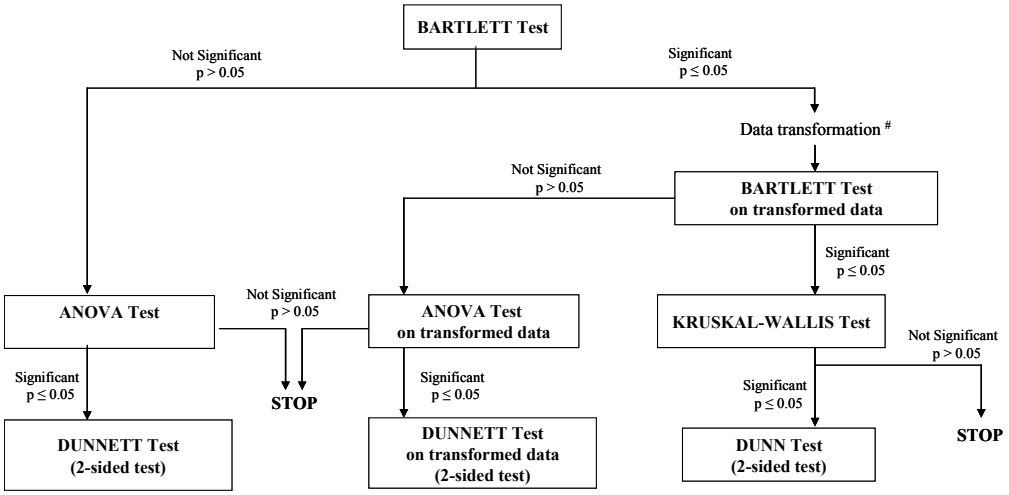

**Phenobarbital: comparison between the treated group and the vehicle control group**




– Body weight and hormonal parameters in the plasma

Mean and standard deviation were calculated for each group.

**Fluopyram: comparison between the treated groups and the vehicle control group**

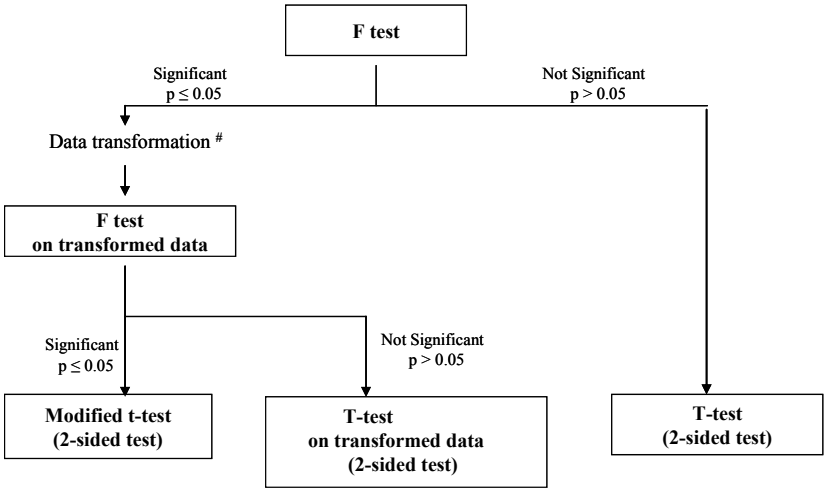



# Data were transformed using the log transformation.

If one or more group variance(s) equal 0, means were compared using non-parametric procedures. Group means were compared at the 5% and 1% levels of significance.

**Phenobarbital: comparison between the treated group and the vehicle control group**

Mean and standard deviation will be calculated for each group




# Data were transformed using the log transformation.

- Hormonal parameters in the bile

T-tests were used for the comparison of T4 bile content in treated groups compared to control group.
Group means were compared at the 5% and 1% levels of significance.
Statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics) except the hormonal measurements in the bile which were performed using Microsoft Excel.

## C. METHODS:

### 1. Observations

The animals were observed twice daily for moribundity and mortality (once daily on weekends or public holidays). Observed clinical signs were recorded at least once daily for all animals and detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility, and duration of any clinical signs were recorded.

### 2. Body weight

All animals were weighed at least once during the acclimatization phase. Each animal was weighed on the first day of test substance administration, then on Day 3.

### 3. Hormonal investigation

**Blood sampling**

On the day of sacrifice, between 8:30 a.m. and 11 a.m., blood samples were taken from all animals from the abdominal aorta. The blood sampling for each animal was performed precisely 24h after the last dose. Animals were anesthetized by inhalation of Isoflurane ([redacted], France) prior to bleeding. Blood was collected on lithium heparin. The resulting plasma samples were stored frozen at approximately -80°C before shipment in dry ice to the subcontracted laboratory

for hormone level evaluation.

**Hormone measurements**

Plasma was prepared from each blood sample and kept frozen at approximately -80°C until the determination of TSH and T4 hormone levels with specific radio-immunoassay kits (supplied by Amersham for TSH and by DIASORIN for T4). This measurement was subcontracted at "Laboratoire d'endocrinologie clinique et toxicologique – 1, [redacted]" under the of the Principal Investigator [redacted].

**4. Sacrifice and pathology**

**Necropsy procedure**

On Study day 4, all designated animals from all groups were sacrificed. All sacrifices were performed by exsanguination under deep anesthesia (inhalation of Isoflurane, [redacted], France). Animals were not diet fasted overnight prior to sacrifice. All animals were necropsied The necropsy included the examination of all major organs, tissues and body cavities. Macroscopic abnormalities were recorded but not sampled except upondecision of the Study Pathologist.

**Tissue collection**

A piece of the median and left liver lobes of 5 animals from each group as well as the thyroid gland (with parathyroid glands) and the pituitary gland of all the animals were sampled. The gall bladder from each animal was collected and pooled in each subgroup by 3 or 4 in eppendorf tubes. The tubes were centrifuged (10000 rpm for 10 minutes) and the supernatants, consisting of bile, were collected for T4 level measurements.

**5. QPCR Analysis:**

## Total RNA purification

Total cytoplasmic RNA was isolated from the pituitary gland of individual control and treated animals using RNeasy Mini kits (Qiagen). RNA quality controls were performed based on the ribosomal RNA electrophoretic profiles using a Bioanalyser (Agilent Technologies).

## Quantitative PCR

Ten µg of total RNA was used for Reverse transcription (RT) using a High Capacity cDNA Archive kit (Applied Biosystems). The assay was performed in duplicate using Taqman assays (Assay on demand, Applied Biosystems), 1/50 diluted first strand cDNA, AmpliTaq Gold® PCR Master Mix on an ABI prism 7900 HT machine (Applied Biosystems). For each gene transcript measured, a negative control condition was included in which H2O MQ was used as template instead of first strand cDNA.

Actin was selected as reference gene for the quantitative calculations of transcripts. The relative quantity (RQ) value of beta subunit Tsh transcript (Tsh b) was calculated using the following formula:

$\Delta\Delta Ct = (Ct_{test} - Ct_{Actin})_{treated} - (Ct_{test} - Ct_{Actin})_{control}$

$RQ = 2^{-\Delta\Delta Ct}$

where Ct is the threshold cycle at which PCR amplification started to be significantly different from the background signal. Each RQ value obtained for a given gene was normalized by dividing by the RQ value obtained for the control animal UT1M4180.

## II. RESULTS AND DISCUSSION

### A. OBSERVATIONS:

Mortality:

There was no mortality during the course of the study.

Clinical signs:

There were no treatment-related clinical signs throughout the duration of the study.

### B. BODY WEIGHT

There was no treatment-related effect on body weight parameters.

### C. HORMONE ANALYSIS:

The magnitude of the changes in the plasma hormone values relative to the controls and statistical significance between treated and control values were as follows:

T4
A statistically significant dose-related decrease in mean T4 levels (-26% and -34% p≤0.01) was observed in the plasma of Fluopyram treated animals. A statistically significant decrease in mean T4 levels (-38%, p≤0.01) was observed in the plasma of Phenobarbital treated animals.

TSH
No statistically significant change in mean TSH levels was observed.

**Table 5.5.4-71: Mean levels of hormones T4 and TSH hormone after exposure to Fluopyram or phenobarbital**

| | **Mean plasma hormone values ± standard deviation (% change compared to control mean values)** | | | |
|---|---|---|---|---|
| | Males | | | |
| Group | 1<br>Control | 2<br>Fluopyram<br>100 mg/kg/day | 3<br>Fluopyram<br>300 mg/kg/day | 4<br>Phenobarbital<br>80 mg/kg/day |
| T4<br>(nmol/l) | 34.2 ± 8.7 | 25.4** ± 6.1<br>(-26%) | 22.6** ± 4.6<br>(-34%) | 21.3** ± 3.1<br>(-38%) |
| TSH<br>(ng/ml) | 3.45 ± 0.40 | 3.37 ± 0.24 | 3.53 ± 0.29 | 3.55 ± 0.37 |

**: The group mean is significantly different from the control at p≤0.01

In the bile, for the mean data, the magnitude of the changes relative to the controls and statistical significance between treated and control values were as follows:

T4

No statistically significant change in mean T4 levels was observed in the bile of Fluopyram treated animals.

An increase of T4 levels in the bile of Phenobarbital treated animals was anticipated as a consequence of an increase of clearance of T4. Paradoxically, a statistically significant decrease in mean T4 levels (-19%, p≤0.01) was observed in the bile of Phenobarbital treated animals. This difference was most likely due to some technical reasons and was considered not to be a treatment related effect.

**Table 5.5.4-72: Mean levels of T4 in the bile after exposure to Fluopyram or phenobarbital**

| | **Mean T4 values in the bile ± standard deviation (% change compared to control mean values)** | | | |
|---|---|---|---|---|
| | Males | | | |
| Group | 1<br>Control | 2<br>Fluopyram<br>100 mg/kg/day | 3<br>Fluopyram<br>300 mg/kg/day | 4<br>Phenobarbital<br>80 mg/kg/day |
| T4 (nmol/l) | 79.2 ± 7.9 | 79.0 ± 6.1 | 83.9 ± 8.5 | 64.5** ± 4.4<br>(-19%) |

**: The group mean is significantly different from the control at p≤0.01

## D. QPCR ANALYSIS

A statistical significant increase of the level of accumulation of Tsh transcript (+49%, p<0.01) was observed in the pituitary gland of Fluopyram treated animals. A statistical significant increase of the level of accumulation of Tsh transcript (+46%, p<0.01) was observed in the pituitary gland of Phenobarbital treated animals.

**Table 5.5.4-73: Tsh b transcript levels after exposure to Fluopyram or phenobarbital**

| | **Mean Relative Quantity ± standard deviation of gene transcripts (% change compared to control mean values)** | | | |
|---|---|---|---|---|
| Gene transcript | Control | 2<br>Fluopyram<br>100 mg/kg/day | 3<br>Fluopyram<br>300 mg/kg/day | 4<br>Phenobarbital<br>80 mg/kg/day |
| Tsh b # | 0.994 ± 0.124 | 1.138 ± 0.230<br>(+21%) | 1.404** ± 0.253<br>(+49%) | 1.381** ± 0.198<br>(+46%) |

# beta subunit Tsh **: p≤0.01

## III. CONCLUSION

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.

| | |
|---|---|
| **Report:** | **KIIA 5.5.4 /27; [redacted] 2012; M-426994-01** |
| Title: | Fluopyram: Mechanistic 3-day toxicity study in the mouse by oral gavage (thyroid hormone investigations) |
| Report No & Document No | SA 10430<br>M-426994-01 |
| **Guidelines:** | **No specific guideline** |
| **Deviations:** | **none** |
| **GLP** | **no** |

**Executive 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.

# I. MATERIALS AND METHODS

## A. MATERIALS:

**1. Test Material:** Fluopyram (AE C656948)
**Description:** Beige powder
**Lot/Batch:** Mix-Batch: 08528/0002
**Purity:** 94.7 %
**CAS:** 658066-35-4
**Stability of test compound:** Stable in 0.5% aqueous solution of methylcellulose for a period covering the study duration

**2. Vehicle and/or positive control:** 0.5% aqueous solution of methylcellulose (vehicle)

**3. Test animals:**
**Species**: Mouse – Male only
**Strain:** C57BL/6J
**Age:** 8 weeks approximately at start of treatment
**Weight at start of dosing:** 19.2 to 25.2 g
**Acclimation period:** 5 days
**Diet:** Certified rodent powdered and irradiated diet A04C-10 P1 from [redacted], *ad libitum*
**Water:** Tap water, *ad libitum*
**Housing:** Animals were caged individually in suspended stainless steel wire mesh cages.
**Environmental conditions –**
**Temperature:** 20 – 24°C
**Humidity:** 40 – 70 %
**Air changes:** Approximately 10-15 changes per hour
**Photoperiod:** Alternating 12-hour light and dark cycles (7 am- 7 pm)

## B. MATERIALS:

**1. In life dates:** Dosing period from January 15 to 20, 2011.

**2. Animal assignment and treatment**

The dose levels for fluopyram were set after evaluation of the results from previous studies conducted with fluopyram and following discussions with the US (EPA) and Canadian (PMRA) authorities. The dose level of 100 mg/kg/day is equivalent to the top dose level (750 ppm) used in the mouse cancer bioassay in which thyroid tumors were observed in the males (SA 05094) and the dose level of 300 mg/kg/day is equivalent to the dose level (2000 ppm) used in previous mouse mechanistic studies conducted with fluopyram (SA 07215, SA 08151 and SA 08288), where thyroid hormone changes were detected.

**Table 5.5.4-74: Study design**

| GROUP | TEST SUBSTANCE | TIME POINT | DOSE LEVEL (mg/kg/day) | NUMBER OF ANIMALS PER GROUP | ANIMAL IDENTITY |
|---|---|---|---|---|---|
| MALES | | | | | |
| 1 | Control | 2h | 0 | 15 | UT1M5032 to 5046 |
| 2 | Fluopyram | 2h | 100 | 15 | UT2M5047 to 5061 |
| 3 | | 2h | 300 | 15 | UT3M5062 to 5076 |
| 4 | Control | 8h | 0 | 15 | UT4M5077 to 5091 |
| 5 | Fluopyram | 8h | 100 | 15 | UT5M5092 to 5106 |
| 6 | | 8h | 300 | 15 | UT6M5107 to 5121 |
| 7 | Control | 14h | 0 | 15 | UT7M5122 to 5136 |
| 8 | Fluopyram | 14h | 100 | 15 | UT8M5137 to 5151 |
| 9 | | 14h | 300 | 15 | UT9M5152 to 5166 |
| 10 | Control | 48h | 0 | 15 | UTXM5167 to 5181 |
| 11 | Fluopyram | 48h | 100 | 15 | UTOM5182 to 5196 |
| 12 | | 48h | 300 | 15 | UTDM5197 to 5211 |

### 3. Dosing preparation and analysis

Fluopyram was suspended in 0.5% aqueous solution of methylcellulose to provide the required concentrations. There was one preparation of the test formulations for the entire study. When not in use the formulations were stored at approximately +4°C.

The stability of Fluopyram at 10 g/l and 30 g/l has been demonstrated in a previous study (SA 04060), which covers the period of storage and usage for the current study.

### 4. Statistics

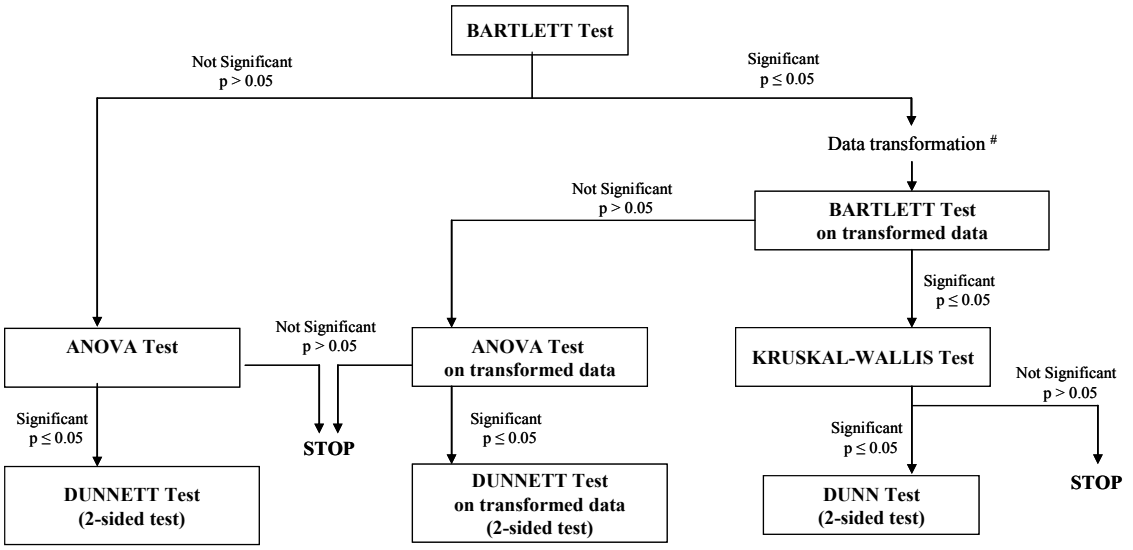
Variables analyzed

- Hormonal parameters in the plasma

Statistical methods

Mean and standard deviation were calculated for each group.

**Fluopyram: comparison between the treated groups and the vehicle control group**




# Data were transformed using the log transformation.

If one or more group variance(s) equal 0, means were compared using non-parametric procedures. Group means were compared at the 5% and 1% levels of significance.

Statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics).

## C. METHODS:

### 1. Observations

The animals were observed twice daily for moribundity and mortality (once daily on weekends or public holidays). Observed clinical signs were recorded at least once daily for all animals and detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility, and duration of any clinical signs were recorded.

### 2. Body weight

All animals were weighed at least once during the acclimatization phase. Each animal was weighed only on the first day of test substance administration.

### 3. Hormonal investigation

**Blood sampling**

On the day of sacrifice, between 8:30 a.m. and 11 a.m., blood samples were taken from all animals from the abdominal aorta. The blood sampling for each animal was performed precisely 2h, 8h, 14h and 48h after the last dose. Animals were anesthetized by inhalation of Isoflurane ([redacted], France) prior to bleeding. Blood was collected on lithium heparin. The resulting plasma samples were stored frozen at approximately -74°C +10°C before shipment in dry ice to the subcontracted laboratory for hormone level evaluation.

**Hormone measurements**
Plasma was prepared from each blood sample and kept frozen at approximately -74°C +10°C until the determination of TSH and T4 hormone levels with specific radio-immunoassay kits (supplied by Amersham for TSH and by DIASORIN for T4). This measurement was subcontracted at "Laboratoire d'endocrinologie clinique et toxicologique – 1, [redacted]" under the of the Principal Investigator [redacted].

## II. RESULTS AND DISCUSSION

### A. OBSERVATIONS:

Mortality:

There was no mortality during the course of the study.

Clinical signs:

There were no treatment-related clinical signs throughout the duration of the study.

### B. HORMONE ANALYSIS:

The magnitude of the changes in the plasma hormone values relative to the controls and statistical significance between treated and control values were as follows:

**T4**
A statistically significant dose-related decrease in mean T4 levels (between -18% and -41%; $p \leq 0.01$) was observed in the plasma of fluopyram treated animals at all time points investigated apart from the 2h time point where the decrease was not dose-related.

**TSH**
No statistically significant change in mean TSH levels was observed at any time point.

**Table 5.5.4-75: Mean levels of hormones T4 and TSH hormone after exposure to Fluopyram**

| TIME POINT | Hormone | Control | Fluopyram (100 mg/kg/day) | Fluopyram (300 mg/kg/day) |
|---|---|---|---|---|
| MALES | | | | |
| 2h | T4 (nmol/l) | 31.5 ± 9.1 | 22.8**± 6.1 (-28%) | 24.0*± 6.3 (-24%) |
| | TSH (ng/ml) | 2.7 ± 0.5 | 2.6 ± 0.5 (-1%) | 2.7 ± 0.5 (+1%) |
| 8h | T4 (nmol/l) | 38.2 ± 7.0 | 28.8** ± 5.4 (-25%) | 22.4** ± 4.1 (-41%) |
| | TSH (ng/ml) | 2.8 ± 0.6 | 3.0 ± 0.5 (+7%) | 3.1 ± 0.7 (+13%) |
| 14h | T4 (nmol/l) | 25.5 ± 5.3 | 20.9** ± 4.5 (-18%) | 18.6** ± 4.3 (-27%) |
| | TSH (ng/ml) | 3.1 ± 0.4 | 3.1 ± 0.7 (+2%) | 3.1 ± 0.5 (+2%) |
| 48h | T4 (nmol/l) | 34.5 ± 8.5 | 25.4** ± 6.1 (-26%) | 24.1** ± 4.4 (-30%) |
| | TSH (ng/ml) | 3.1 ± 0.6 | 3.2 ± 0.5 (+3%) | 3.2 ± 0.6 (+2%) |

*: The group mean is significantly different from the control at p≤0.05
**: The group mean i s significantly different from the control at p≤0.01

## III. CONCLUSION

In the fluopyram treated animals, after 3 days of exposure, at 100 or 300 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≤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.

| | |
|---|---|
| **Report:** | KIIA 5.5.4 /28; [redacted] 2012; M-428031-02; Amended: 2013-03-22 |
| Title: | Fluopyram: Mechanistic 28-day toxicity study in the mouse by dietary administration (hepatotoxicity and thyroid hormone investigations) |
| Report No & Document No | SA 11105<br>M-428031-02 |
| **Guidelines:** | **No specific guideline** |
| **Deviations:** | **none** |
| **GLP** | **yes** |

**Executive 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 were 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%; $p \leq 0.01$) 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, anincrease 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 atterminal sacrifice. At necropsy, statistically significant increases in absolute mean liver weight andmean 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. Nonstatistically 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 ( $p \leq 0.05$). 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.

## I. MATERIALS AND METHODS

### A. MATERIALS:

**1. Test Material:** Fluopyram (AE C656948)
- **Description:** Beige powder
- **Lot/Batch:** Mix-Batch: 08528/0002
- **Purity:** 94.7 %
- **CAS:** 658066-35-4
- **Stability of test compound:** Stable in rodent diet for a period covering the study duration

**2. Vehicle and/or positive control:** Vehicle for AE C656948: rodent diet
Phenobarbital (positive control), vehicle methylcellulose
- **Description:** White crystalline powder
- **Lot/Batch:** Lot No. 09050075
- **Purity:** 100 %
- **CAS:** 50-06-6
- **Stability of test compound:** Stable in 0.5% aqueous solution of methylcellulose for a period covering the study duration

**3. Test animals:**
- **Species**: Mouse – Male only
- **Strain:** C57BL/6J
- **Age:** 8 weeks approximately at start of treatment
- **Weight at start of dosing:** 18.1 to 24.7 g
- **Acclimation period:** 13 to 15 days
- **Diet:** Certified rodent powdered and irradiated diet A04C-10 P1 from [redacted] ([redacted], [redacted], France), *ad libitum*
- **Water:** Tap water, *ad libitum*
- **Housing:** Animals were caged individually in suspended stainless steel wire mesh cages.

**Environmental conditions –**

| | |
|---|---|
| **Temperature:** | 20 – 24°C |
| **Humidity:** | 40 – 70 % |
| **Air changes:** | Approximately 10-15 changes per hour |
| **Photoperiod:** | Alternating 12-hour light and dark cycles (7 am- 7 pm) |

## B. STUDY DESIGN:

**1. In life dates:** Dosing period from May 24 to June 23, recovery period from June 22 to July 25 2011.

**2. Animal assignment and treatment**

Seven groups of male mice were dosed for at least 28 days with either control diet or the appropriate compound by the appropriate route of administration.

Six groups were dosed by dietary administration. One group consisted of control animals that received untreated diet and the remaining five groups received fluopyram at the appropriate dietaryconcentration (30, 75, 150, 600 and 750 ppm) at a constant level. The dose levels for fluopyramwere set after evaluation of the results from previous studies and following discussions with the US (EPA) and Canadian (PMRA) authorities. The top dose level of 750 ppm used in the present study represents the top dose level used in the mouse cancer bioassay in which thyroid tumors wereobserved in the males (SA 05094).

The seventh group was dosed by oral gavage with 80 mg/kg/day phenobarbital suspended in 0.5%aqueous methylcellulose 400 using a dosing volume of 5 ml/kg bodyweight the first week of treatment and then using a dosing volume of 10 ml /kg bodyweight for the remaining treatment period. The volume administered to each mouse was based on the most recent recorded body weight. A dose level of 80 mg/kg/day was chosen for phenobarbital as this dose was used in previous mouse mechanistic studies conducted with phenobarbital, where thyroid changes were detected (SA 07326, SA 08151, SA 08288).

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 control or test diet (750 ppm fluopyram) or were orally dosed (80 mg/kg/day phenobarbital) for at least 28 days and were then allowed one month (at least 28 days) of recovery during which they were maintained on untreated control diet.

All animals were sacrificed in the morning after the last day of treatment or after the last day of the recovery phase.

**Table 5.5.4-76: Study design**

| GROUP | TEST SUBSTANCE | DOSE LEVEL | NUMBER OF ANIMALS PER GROUP | ANIMAL IDENTITY |
|---|---|---|---|---|
| MALES | | | | |
| 1 | Control | 0 | 15 + 15* | T1M1885 to 1914 |
| 2 | Fluopyram | 30 ppm (5 mg/kg/day) | 15 | T2M1915 to 1929 |
| 3 | | 75 ppm (13 mg/kg/day) | 15 | T3M1930 to 1944 |
| 4 | | 150 ppm (25 mg/kg/day) | 15 | T4M1945 to 1959 |
| 5 | | 600 ppm (102 mg/kg/day) | 15 | T5M1960 to 1974 |
| 6 | | 750 ppm (128 mg/kg/day) | 15 + 15* | T6M1975 to 2004 |
| 7 | Phenobarbital | 80 mg/kg/day | 15 + 15* | T7M2005 to 2034 |

- Recovery phase

**3. Diet preparation and analysis**

Fluopyram was incorporated into the diet to provide the required dietary concentrations.
The test formulations were stored at room temperature and issued to the animal unit in polyethylene containers. There was one formulation for each concentration used in the study and any unused diets were discarded at the end of each administration period.

The homogeneity of fluopyram in diet was verified at the lowest and highest concentrations. The mean values obtained from the homogeneity check were taken as measured concentrations. For the remaining concentrations, the dietary levels were verified for each concentration at the time of preparation. The homogeneity and concentration data for fluopyram in rodent diet and was within a range of 89 and 98% of the nominal concentration. Therefore all values were within the in-house target range of 85-115% of the nominal concentration. The stability of fluopyram at 20 and 10000 ppm in the diet was demonstrated in an earlier study (SA 03332), which covered the period of storage and usage for the present study.

Phenobarbital formulations were prepared twice during the study by suspending the test substance (w/w) in a 0.5% aqueous solution of methylcellulose 400. The method of preparation was documented in the study file. This suspension was stored in air-tight bottles at +5°C ± 3°C when not in use. The suspension was mixed continuously before and during dosing using an electromagnetic stirrer. Any unused formulation was discarded at the end of the administration period.

The homogeneity of the first phenobarbital formulations at 16 and 8 g/l were verified. The sampling was done at two levels at the surface and at the bottom. The mean values obtained from the homogeneity checks were used as measured concentrations. For the second formulation, the sampling was done at the surface and the mean value obtained from the sampling was used as measured concentration. The homogeneity and concentration data for phenobarbital as an aqueous suspension in 0.5% methylcellulose 400 were between 93 and 101% of the nominal concentration. Therefore all values were within the in-house target range of 90-110% of the nominal concentration. The stability of phenobarbital at 8 g/l has been demonstrated in a previous study (SA 03310), which covers the period of storage and usage for the current study.

## 4. Statistics

Variables analyzed

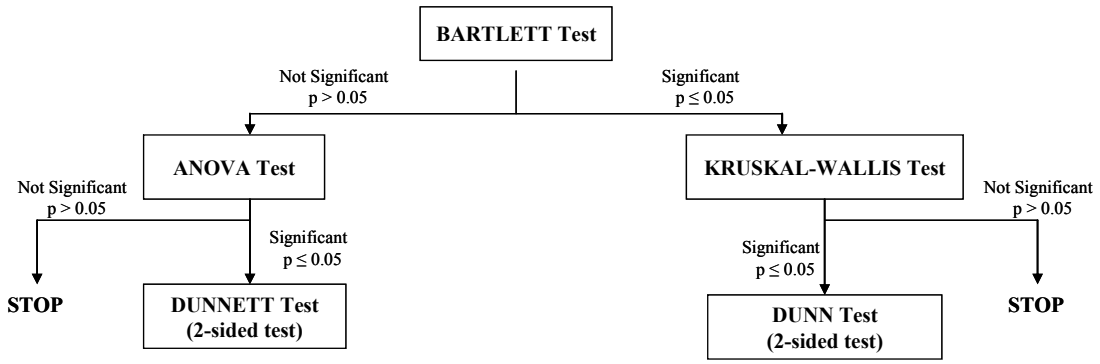
- Body weight parameters
- Body weight gain/day parameters calculated according to time intervals
- Average food consumption/day parameters calculated according to time intervals
- Hormonal parameters
- Terminal body weight, absolute and relative organ weights parameters
- Liver enzyme activities
- qPCR determinations

Statistical methods

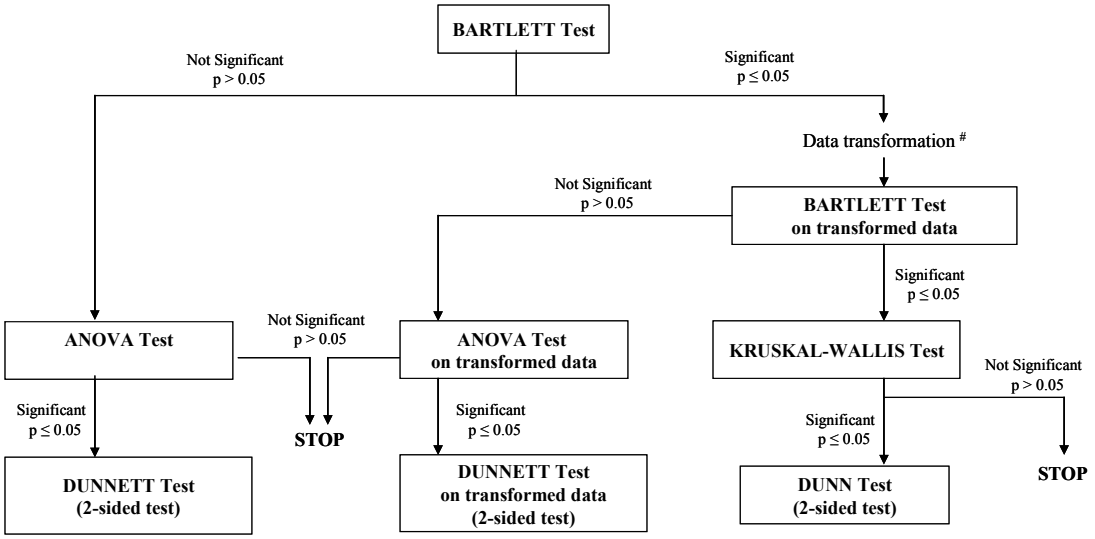
Mean and standard deviation were calculated for each group.

**Fluopyram: comparison between the treated groups and the vehicle control group**

– Body weight change parameters,
– Terminal body weight, absolute and relative organ weight parameters,




– Body weight and average food consumption/day parameters
– Hormonal parameters
– Liver enzyme activities
– qPCR determinations




# Data was transformed using the log transformation.

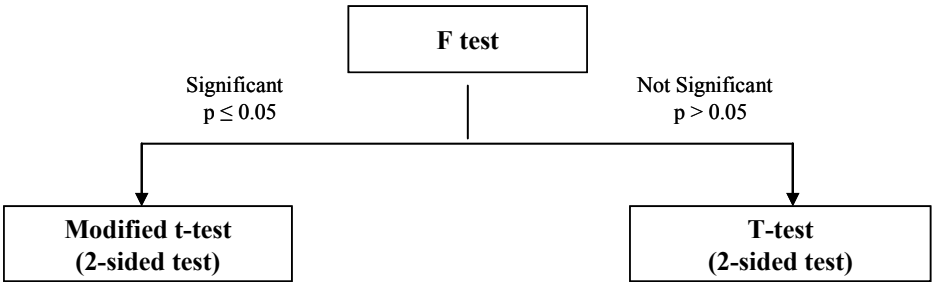
If one or more group variance(s) equal 0, means will be compared using non-parametric procedures.

Group means were compared at the 5% and 1% levels of significance.

## Phenobarbital: comparison between the treated group and the vehicle control group

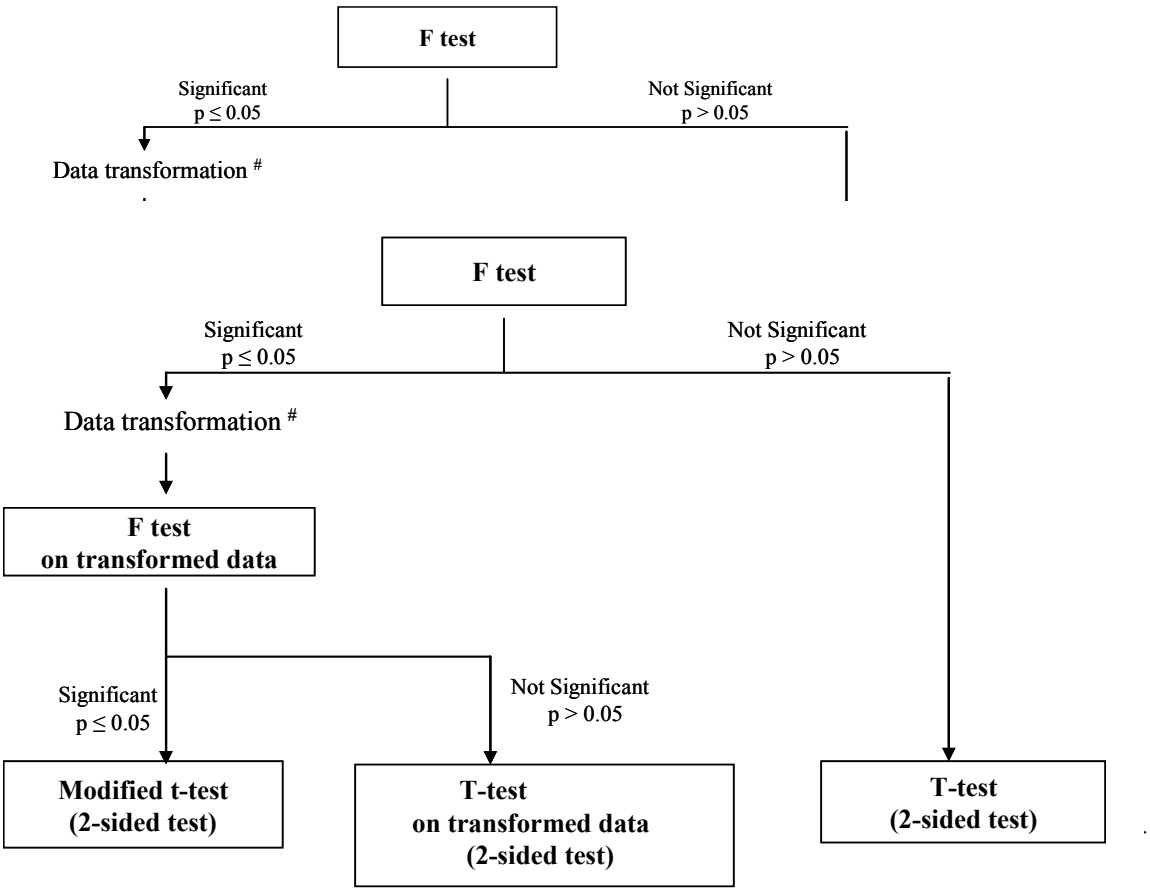
- Body weight change parameters,
- Terminal body weight, absolute and relative organ weight parameters,

Mean and standard deviation was calculated for each group and per time period for body weight change parameters




- Body weight and average food consumption/day parameters
- Hormonal parameters
- Liver enzyme activities
- qPCR determinations

Mean and standard deviation were calculated for each group and per time period for average food consumption/day parameters.




## C. METHODS:

### 1. Observations

The animals were observed twice daily for moribundity and mortality (once daily on weekends or public holidays). Observed clinical signs were recorded at least once daily for all animals and detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility, and duration of any clinical signs were recorded.

### 2. Body weight

Each animal was weighed at least once during the acclimatization phase, on study Day 1 and then weekly during the treatment period and recovery phase. Additionally, animals were weighed before scheduled necropsy (terminal body weight).

### 3. Food consumption and compound intake

The weight of food supplied and of that remaining at the end of the food consumption period was recorded weekly for all animals during the treatment period to calculate the mean daily food consumption. Any food spillage was noted. From these records, the weekly mean achieved dosage intake in mg/kg/day for fluopyram for weeks 1 to 4 was calculated using the following formula:

$$\underset{\text{(mg/kg/day)}}{\text{Test substance intake}} = \frac{\text{Dose level (ppm)} \times \text{Group mean food consumption (g/day)}}{\text{Group mean body weight (g) at the end of the food consumption period}}$$

**4. Hormonal investigation**

**Blood sampling**
On the day of sacrifice, between 8:30 a.m. and 11 a.m., blood samples were taken from all animals from the abdominal aorta. Animals were anesthetized by inhalation of Isoflurane ([redacted], France) prior to bleeding. Blood was collected on lithium heparin. The resulting plasma samples were frozen at approximately -74°C + 10°C before shipment in dry ice to the subcontracted laboratory for hormone measurement.

**Hormone measurements**
Plasma was prepared from each blood sample and kept frozen at approximately -74°C + 10°C until the determination of TSH and T4 hormone levels with specific radio-immunoassay kits (supplied by Amersham for TSH and by DIASORIN for T4). This measurement was subcontracted at " [redacted], [redacted], France "

**5. Sacrifice and pathology**

**Necropsy procedure – Organ sampling**
All designated animals from all groups were sacrificed on Days 29 and 30 following treatment or on Day 29 following the recovery phase . All sacrifices were performed by exsanguination under deep anesthesia (inhalation of Isoflurane, [redacted], France). Animals were not diet fasted overnight prior to sacrifice. All animals were necropsied. The necropsy included the examination of all major organs, tissues and body cavities. Macroscopic abnormalities were recorded but not sampled except upon decision of the Study Pathologist.

A piece of the median and left liver lobes of 5 animals from each group as well as the pituitary gland of all the animals were sampled.

For each group, the pituitary gland was collected and flash frozen in liquid nitrogen. These samples were stored individually at approximately -74°C + 10°C until used for qPCR investigations.

**6. Hepatotoxicity testing**

At each sacrifice time, the livers were weighed, pooled by three in each treatment group and homogenized for microsomal preparations to determine UDPGT specific isoenzyme activities.

**QPCR Analysis**

**Total RNA purification**

Total cytoplasmic RNA was isolated from the pituitary gland of individual control and treated animals using RNeasy Mini kits (Qiagen). RNA quality controls were performed based on the ribosomal RNA electrophoretic profiles using a Bioanalyser (Agilent Technologies).

**Quantitative PCR**

One µg of total RNA was used for Reverse transcription (RT) using a High Capacity cDNA Archive kit (Applied Biosystems). The assay was performed in duplicate using a beta subunit Tsh Taqman assay (Assay on demand, Applied Biosystems, ref Mm03990915-g1), 1/25 diluted first strand cDNA, FastStart Universal Probe Master Mix (Roche) on an ABI prism 7900 HT machine (Applied Biosystems). For each gene transcript measured, a negative control condition was included in which H2O MQ was used as template instead of first strand cDNA. B-microtubulin, beta Actin, Gusb, and Gapdh were tested as reference genes. For the quantitative calculations of Tsh transcripts, Gusb was selected as reference gene. The relative quantity (RQ) value of beta subunit Tsh transcript (Tsh b) was calculated using the following formula:

$\Delta\Delta Ct$ = (CtTsh b – CtGusb) treated - (CtTsh b – CtGusb) control

$RQ = 2^{-\Delta\Delta Ct}$

where Ct is the threshold cycle at which PCR amplification started to be significantly different from the background signal.Each RQ value obtained for a given gene was normalized by dividing by the RQ value obtained for the control animal T1M1885 for the dosing phase and T1M1900 for the recovery phase.

**UDPGT activities**

UDPGT activity with bilirubin as substrate was determined using a spectrophotometry method consisting in the determination of conjugated bilepigments after their conversion into azo-pigment derivatives. Absorbance was measured at 530 nm. Three replicates from each sample were assayed.

The glucuronidation of the substrate T4 was subcontracted to [redacted], UK.

## II. RESULTS AND DISCUSSION

### A. OBSERVATIONS:

Mortality:

Fluopyram: There was no mortality in any groups throughout the study.

Phenobarbital: Animal T7M2020 was sacrificed for humane reasons on study day 2 and two animals (T7M2030 and T7M2033) were found dead on study day 5 and 7, respectively. 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. Therefore, a concentration of 8 g/l at a dosing volume of 10 ml /kg body weight was administered for the rest of the dosing period.

Clinical signs:

*Dosing phase*

Fluopyram: There were no treatment-related clinical signs.

Phenobarbital: All males displayed reduced motor activity during the dosing phase.

*Recovery phase*

Fluopyram: There were no treatment-related clinical signs.

Phenpbarbital: There were no treatment-related clinical signs.

**B. BODY WEIGHT AND BODY WEIGHT GAIN:**

*Dosing phase*

Fluopyram: There was no effect on body weight parameters.

Phenobarbital: Body weight and body weight gain parameters were statistically significantly reduced throughout the treatment period, resulting in a 5% ($p \leq 0.01$) reduction in body weight and a 42% ($p \leq 0.01$) reduction in cumulative body weight gain by Day 29.

*Recovery phase*

Fluopyram: There was no effect on body weight parameters.

Phenobarbital: Body weight was slightly reduced at the start of the recovery period (-5%; $p \leq 0.05$), compared to the control group, but was only marginally lower than the control group by Day 29 (-3%; not statistically significant).

**C. FOOD CONSUMPTION:**

*Dosing phase*

Fluopyram: Food consumption was unaffected by treatment throughout the dosing phase.

Phenobarbital: Food consumption was considered to be comparable to the control group throughout the dosing phase, even though consumption was marginally reduced during the first week.

*Recovery phase*

Fluopyram: Food consumption was comparable to the control group throughout the recovery phase.

Phenobarbital: Food consumption was considered to be comparable to the control group throughout the recovery phase, even though consumption was marginally reduced during Week 4.

**D. HORMONE ANALYSIS:**

The magnitude of the changes in the plasma hormone values relative to the controls and statistical significance between treated and control values were as follows:

*Dosing phase*

Fluopyram:

**T4**
A statistically significant decrease in mean T4 levels ($p \leq 0.01$) was observed at all the dose levels

in the plasma of fluopyram treated animals when compared to controls, though not in a clear dose-related manner.

**TSH**
No statistically significant change in mean TSH levels was observed.

**Table 5.5.4-77: Mean levels of T4 and TSH hormones in the plasma after exposure to Fluopyram**

| Mean plasma hormone values ± standard deviation (% change compared to control mean values) | | | | | | |
|---|---|---|---|---|---|---|
| Dose-level (ppm) | Fluopyram | | | | | |
| | 0 | 30 | 75 | 150 | 600 | 750 |
| T4 (nmol/l) | 26.1 ±6.9 | 18.9** ±3.1 (-28%) | 17.9** ±3.4 (-31%) | 19.5** ±4.6 (-25%) | 16.5** ±2.3 (-37%) | 16.3** ±2.9 (-38%) |
| TSH (ng/ml) | 1.4 ±0.6 | 2.1 ±0.9 (+50%) | 1.6 ±0.5 (+14%) | 1.2 ±0.3 (-14%) | 1.6 ±0.7 (+14%) | 1.6 ±0.6 (+14%) |

**: p≤0.01

Phenobarbital:

**T4**
A statistically significant decrease in mean T4 levels (-23%, p≤0.01) was observed in the plasma of phenobarbital treated animals.

**TSH**
No statistically significant change in mean TSH levels was observed.

**Table 5.5.4-78: Mean levels of T4 and TSH hormones in plasma after exposure to Phenobarbital**

| Mean plasma hormone values ± standard deviation (% change compared to control mean values) | | |
|---|---|---|
| | Phenobarbital | |
| | 0 ppm | 80 mg/kg |
| T4 (nmol/l) | 26.1 ±6.9 | 20.1**±2.7 (-23%) |
| TSH (ng/ml) | 1.4 ±0.6 | 1.6±0.4 (+14%) |

**: p≤0.01

*Recovery Phase:*

Fluopyram**:**

**T4**
No statistically significant change in mean T4 levels was observed.

**TSH**
No statistically significant change in mean TSH levels was observed.

**Table 5.5.4-79: Mean levels of T4 and TSH hormones in plasma after exposure to Fluopyram followed by recovery period**

| Mean plasma hormone values ± standard deviation (% change compared to control mean values) | | |
|---|---|---|
| Dose-level (ppm) | Fluopyram | |
| | 0 | 750 |
| T4 (nmol/l) | 28.5 ±5.4 | 27.1±5.6 (-5%) |
| TSH (ng/ml) | 1.5 ±0.3 | 1.4±0.4 (-7%) |

Phenobarbital:

**T4**
No statistically significant change in mean T4 levels was observed.

**TSH**
No statistically significant change in mean TSH levels was observed.

**Table 5.5.4-80: Mean levels of T4 and TSH hormones after exposure to Phenobarbital followed by recovery period**

| Mean plasma hormone values ± standard deviation (% change compared to control mean values) | | |
|---|---|---|
| | Phenobarbital | |
| | 0 ppm | 80 mg/kg |
| T4 (nmol/l) | 28.5±5.4 | 28.4 ±7.5 (-0.4%) |
| TSH (ng/ml) | 1.5 ±0.3 | 1.6 ±0.5 (+7%) |

## E. QPCR ANALYSIS:

*Dosing phase*

Fluopyram

A dose-related increase in the level of accumulation of Tsh transcript in the pituitary gland was recorded at 600 and 750 ppm (+43%; p≤0.05; +54%; p≤0.01).

**Table 5.5.4-81: Tsh b transcript levels after exposure to Fluopyram**

| Mean relative quantity ± standard deviation of Tsh b transcript (% change compared to control mean values) | | | | | | |
|---|---|---|---|---|---|---|
| Dose-level (ppm) | Fluopyram | | | | | |
| | 0 | 30 | 75 | 150 | 600 | 750 |
| Tsh b | 1.156 ±0.334 | 1.223 ±0.354 (+6%) | 1.303 ±0.344 (+13%) | 1.297 ±0.451 (+12%) | 1.655* ±0.461 (+43%) | 1.783** ±0.725 (+54%) |

**: p≤0.01

Phenobarbital

An increase in the level of accumulation of Tsh transcript in the pituitary gland was recorded in phenobarbital treated animals (+53%; p≤0.01).

**Table 5.5.4-82: Tsh b transcript levels after exposure to Phenobarbital**

| Mean relative quantity ± standard deviation of Tsh b transcript (% change compared to control mean values) | | |
|---|---|---|
| | Phenobarbital | |
| | 0 ppm | 80 mg/kg |
| Tsh b | 1.156 ±0.334 | 1.764** ±0.586 (+53%) |

**: p≤0.01

*Recovery Phase:*

Fluopyram:

A slight increase in the level of accumulation of Tsh transcript in the pituitary gland was recorded in fluopyram treated animals after the recovery phase (+12%; p≤0.05).

**Table 5.5.4-83: Tsh b transcript levels after exposure to Fluopyram followed by recovery phase**

| Mean relative quantity ± standard deviation of Tsh b transcript (% change compared to control mean values) | | |
|---|---|---|
| Dose-level (ppm) | Fluopyram | |
| | 0 | 750 |
| Tsh b | 1.046 ±0.087 | 1.169* ±0.184 (+12%) |

*: p≤0.05

Phenobarbital

No statistically significant change in the level of accumulation of Tsh transcript in the pituitary gland was recorded in phenobarbital treated animals after the recovery phase.

**Table 5.5.4-84: Tsh b transcript levels after exposure to Phenobarbital followed by recovery phase**

| Mean relative quantity ± standard deviation of Tsh b transcript (% change compared to control mean values) | | |
|---|---|---|
| | Phenobarbital | |
| | 0 ppm | 80 mg/kg |
| Tsh b | 1.046 ±0.087 | 1.090 ±0.139 (+4%) |

## F. POST MORTEM EXAMINATIONS:

**Terminal body weight and organ weight**

*Dosing phase*

Fluopyram:

There was no change in mean terminal body weight in treated animals when compared to the controls.

At 75 ppm, mean liver to body weight ratio was statistically significantly higher when compared to controls. From 150 ppm, mean absolute and relative liver weight were statistically significantly higher when compared to controls.

These changes were considered to be treatment-related.

**Table 5.5.4-85: Mean liver weight changes after exposure to Fluopyram**

| MEAN LIVER WEIGHT ±SD AT SCHEDULED SACRIFICE (% CHANGE WHEN COMPARED TO CONTROLS) | | | | | | |
|---|---|---|---|---|---|---|
| Dose-level (ppm) | Fluopyram | | | | | |
| | 0 | 30 | 75 | 150 | 600 | 750 |
| Absolute liver weight (g) | 1.31 ±0.08 | 1.37 ±0.13 (-5%) | 1.40 ±0.09 (+7%) | 1.45** ±0.13 (+11%) | 1.67** ±0.12 (+27%) | 1.78** ±0.12 (+36%) |
| Liver to body weight ratio (%) | 5.338 ±0.310 | 5.570 ±0.306 (+4%) | 5.667* ±0.278 (+6%) | 5.825** ±0.275 (+9%) | 6.786** ±0.468 (+27%) | 7.088** ±0.341 (+33%) |

*: p≤0.05; **: p≤0.01

Phenobarbital:

There was no change in mean terminal body weight in treated animals when compared to the controls.

Mean absolute and relative liver weight were statistically significantly higher when compared to controls following phenobarbital treatment.

**Table 5.5.4-86: Mean liver weight changes after exposure to Phenobarbital**

| MEAN LIVER WEIGHT ±SD AT SCHEDULED SACRIFICE (% CHANGE WHEN COMPARED TO CONTROLS) | | |
|---|---|---|
| | Phenobarbital | |
| | 0 ppm | 80 mg/kg |
| Absolute liver weight (g) | 1.31 ±0.08 | 1.44** ±0.12 (+10%) |
| Liver to body weight ratio (%) | 5.338 ±0.310 | 6.190** ±0.291 (+16%) |

**: p≤0.01

*Recovery Phase:*

Fluopyram:

There was no change in mean terminal body weight in treated animals when compared to the controls.

There was no significant change in liver weight parameters in treated animals when compared to the controls.

**Table 5.5.4-87: Mean liver weight changes after exposure to Fluopyram followed by recovery period**

| MEAN LIVER WEIGHT ±SD AT SCHEDULED SACRIFICE (% CHANGE WHEN COMPARED TO CONTROLS) | | |
|---|---|---|
| Dose-level (ppm) | Fluopyram | |
| | 0 | 750 |
| Absolute liver weight (g) | 1.32 ±0.10 | 1.37 ±0.18 (+4%) |
| Liver to body weight ratio (%) | 4.984 ±0.296 | 5.190 ±0.500 (+4%) |

Phenobarbital:

There was no change in mean terminal body weight in treated animals when compared to the controls.

There was no significant change in liver weight parameters in treated animals when compared to the controls.

**Table 5.5.4-88: Mean liver weight changes after exposure to Phenobarbital followed by recovery period**

| MEAN LIVER WEIGHT ±SD AT SCHEDULED SACRIFICE (% CHANGE WHEN COMPARED TO CONTROLS) | | |
|---|---|---|
| | Phenobarbital | |
| | 0 ppm | 80 mg/kg |
| Absolute liver weight (g) | 1.32 ±0.10 | 1.33 ±0.09 (+1%) |
| Liver to body weight ratio (%) | 4.984 ±0.296 | 5.085 ±0.282 (+2%) |

## G. UDPGT ACTIVITIES:

*Dosing phase*

Fluopyram:

A dose-related increase in the UDPGT enzymatic activity using bilirubin as substrate was recorded at 600 and 750 ppm (+39%; $p \leq 0.05$; +48%; $p \leq 0.01$).
An increase in the UDPGT enzymatic activity using thyroxine as substrate was recorded at 600 ppm (+83%; $p \leq 0.01$). An increase of this activity was also detected at 150 and 750 ppm, although not statistically significant.

**Table 5.5.4-89: Mean enzymatic UDPGT activities after exposure to Fluopyram**

| Mean enzymatic UDPGT activities ± standard deviation (% change compared to control mean values) | | | | | | |
|---|---|---|---|---|---|---|
| Dose-level (ppm) | Fluopyram | | | | | |
| | 0 | 30 | 75 | 150 | 600 | 750 |
| UDPGT (Bilirubin) | 1.985±0.577 | 2.217 ±0.375 (+12%) | 2.394 ±0.327 (+21%) | 2.624 ±0.198 (+32%) | 2.758* ±0.324 (+39%) | 2.947** ±0.422 (+48%) |
| UDPGT (Thyroxine) | 0.770±0.144 | 0.768 ±0.190 (0%) | 0.854 ±0.198 (+11%) | 1.168 ±0.360 (+52%) | 1.412** ±0.253 (+83%) | 1.026 ±0.271 (+33%) |

*: p≤0.05; **: p≤0.01

Phenobarbital:

Non-statistically significant increases of the hepatic UDPGT enzymatic activities (+42% and +32%) were recorded in the phenobarbital treated animals.

**Table 5.5.4-90: Mean enzymatic UDPGT activities after exposure to Phenobarbital**

| Mean enzymatic UDPGT activities ± standard deviation (% change compared to control mean values) | | |
|---|---|---|
| | Phenobarbital | |
| | 0 ppm | 80 mg/kg |
| UDPGT (bilirubin) | 1.985±0.577 | 2.821±0.676 (+42%) |
| UDPGT (Thyroxine) | 0.770±0.144 | 1.020±0.210 (+32%) |

*Recovery phase*

Fluopyram:

There was no significant change in the hepatic UDPGT enzymatic activities in the fluopyram treated animals after the recovery phase.

**Table 5.5.4-91: Mean enzymatic UDPGT activities after exposure to Fluopyram followed by recovery period**

| Mean enzymatic UDPGT activities ± standard deviation (% change compared to control mean values) | | |
|---|---|---|
| Dose-level (ppm) | Fluopyram | |
| | 0 | 750 |
| UDPGT (bilirubin) | 1.795±0.313 | 1.908±0.237 (+6%) |
| UDPGT (Thyroxine) | 0.822±0.115 | 0.774±0.327 (-6%) |

Phenobarbital:

There was no significant change in the hepatic UDPGT enzymatic activities in the phenobarbital treated animals after the recovery phase.

**Table 5.5.4-92: Mean enzymatic UDPGT activities after exposure to Phenobarbital followed by recovery period**

| Mean enzymatic UDPGT activities ± standard deviation (% change compared to control mean values) | | |
|---|---|---|
| | Phenobarbital | |
| | 0 ppm | 80 mg/kg |
| UDPGT (bilirubin) | 1.795±0.313 | 1.992±0.293 (+11%) |
| UDPGT (Thyroxine) | 0.822±0.115 | 0.808±0.096 (-2%) |

## III. CONCLUSION

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.

| | |
|---|---|
| **Report:** | **KIIA 5.5.4 /29; [redacted] 2012; M-428303-01** |
| Title: | Fluopyram: 28-day toxicity study for proliferation assessment in the C57BL/6J male mouse |
| Report No & | SA 11123 |
| Document No | M-428303-01 |
| **Guidelines:** | **No specific guideline** |
| **Deviations:** | **none** |
| **GLP** | **yes** |

**Executive 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≤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≤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.

## I. MATERIALS AND METHODS

### A. MATERIALS:

**1. Test Material:** Fluopyram (AE C656948)
- **Description:** Beige powder
- **Lot/Batch:** Mix-Batch: 08528/0002
- **Purity:** 94.7 %
- **CAS:** 658066-35-4
- **Stability of test compound:** Stable in rodent diet for a period covering the study duration

**2. Vehicle and/or positive control:** Vehicle for AE C656948: rodent diet
Phenobarbital (positive control)
Vehicle for Phenobarbital: methylcellulose
- **Positive control:** Phenobarbital
- **Description:** White crystalline powder
- **Lot/Batch:** Lot No. 09050075
- **Purity:** 100 %
- **CAS:** 50-06-6
- **Stability of test compound:** Stable in 0.5% aqueous solution of methylcellulose for a period covering the study duration

**3. Test animals:**
- **Species**: Mouse – Male only
- **Strain:** C57BL/6J
- **Age:** 8 weeks approximately at start of treatment
- **Weight at start of dosing:** 19.6 to 24.8 g
- **Acclimation period:** 14 days
- **Diet:** Certified rodent powdered and irradiated diet A04C-10 P1 from [redacted], France), *ad libitum*
- **Water:** Tap water, *ad libitum*
- **Housing:** Animals were caged individually in suspended stainless steel wire mesh cages.
- **Environmental conditions –**
  - **Temperature:** 20 – 24°C
  - **Humidity:** 40 – 70 %

**Air changes:** Approximately 10-15 changes per hour
**Photoperiod:** Alternating 12-hour light and dark cycles (7 am- 7 pm)

## B. STUDY DESIGN:

**1. In life dates:** Dosing period from June 8 to July 6, 2011.

**2. Animal assignment and treatment**

Three groups of male mice were dosed for at least 28 days with the appropriate compound by the appropriate route of administration. Each group consisted of 15 male mice.

Two groups were dosed by dietary administration. One group consisted of control animals that received untreated diet and the second group received fluopyram at 750 ppm at a constant level. The level for fluopyram was set after evaluation of the results from a previous study conducted with fluopyram (SA 10241) and following discussions with the US (EPA) and Canadian (PMRA) authorities. The level of 750 ppm used in the present study represents the highest level used in the mouse cancer bioassay in which an increase in thyroid tumors was observed in the males (SA 05094).

The third group was dosed by oral gavage with 80 mg/kg/day Phenobarbital suspended in 0.5% aqueous methylcellulose 400 using a dosing volume of 10 ml/kg bodyweight. The volume administered to each mouse was based on the most recent recorded body weight. A dose level of 80 mg/kg/day was chosen for phenobarbital as this dose was used in a previous mouse mechanistic study conducted with phenobarbital, where thyroid hormone level change was detected (SA 07326).

**Table 5.5.4-.93-: Study design**

| GROUP | TEST SUBSTANCE | DOSE LEVEL | NUMBER OF ANIMALS PER GROUP | ANIMAL IDENTITY |
|---|---|---|---|---|
| MALES | | | | |
| 1 | Control | 0 ppm | 15 | T1M2166 to 2180 |
| 2 | Fluopyram | 750 ppm (127 mg/kg/day) | 15 | T2M2181 to 2195 |
| 3 | Phenobarbital | 80 mg/kg/day | 15 | T3M2196 to 2210 |

**3. Diet preparation and analysis**

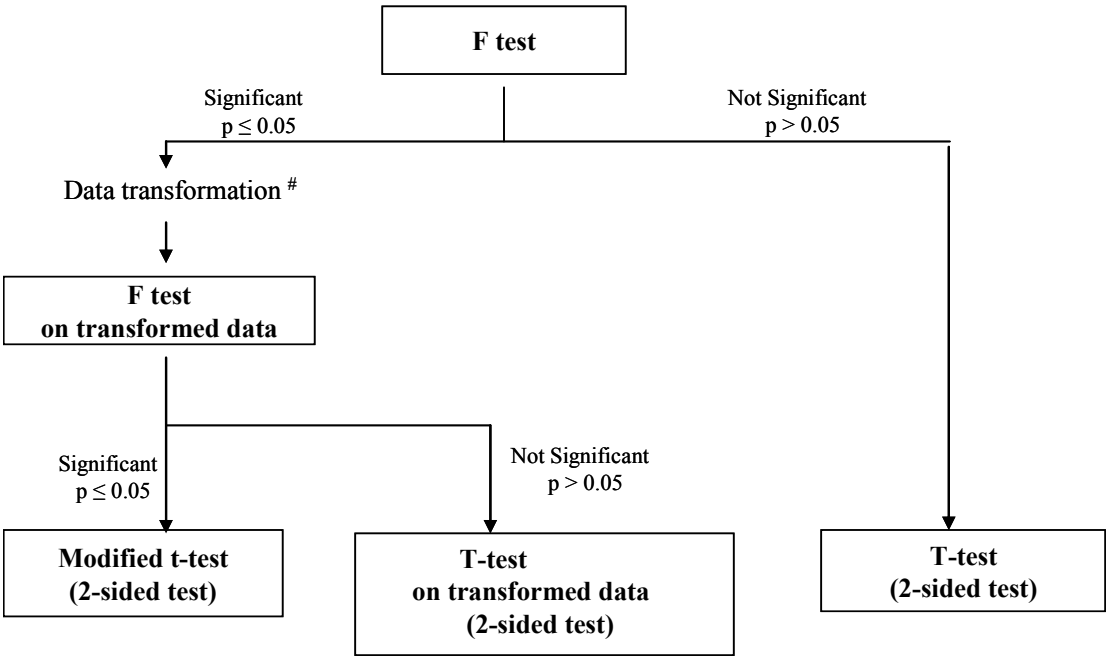
Fluopyram was incorporated into the diet to provide the required dietary concentrations.
The test formulation was stored at room temperature and issued to the animal unit in polyethylene containers. There was one formulation used in the study and any unused diet was discarded at the end of each administration period.

The homogeneity of fluopyram in diet was verified, the mean values obtained from the homogeneity check was taken as measured concentration. For the remaining concentrations, the dietary levels were verified for each concentration at the time of preparation. The homogeneity and concentration data for fluopyram in rodent diet were between 98 and 99% of the nominal concentration. Therefore the values were within the in-house target range of 85-115% of the nominal concentration. The stability of fluopyram at 20 and 10000 ppm in the diet was

demonstrated in an earlier study (SA 03332), which covered the period of storage and usage for the present study.

Phenobarbital formulations were prepared twice during the study by suspending the test substance (w/w) in a 0.5% aqueous solution of methylcellulose 400. This suspension was stored in air-tight bottles at +5°C ± 3°C when not in use. The suspension was mixed continuously before and during dosing using an electromagnetic stirrer. Any unused formulation was discarded at the end of the administration period.

The homogeneity of phenobarbital in aqueous 0.5% methylcellulose 400 suspension was verified on first formulation to demonstrate adequate formulation preparation. The mean value obtained




Group means were compared at the 5% and 1% levels of significance.

With the exception of those used for the cell proliferation data, all statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics). SAS programs (version 9.2) were used for the cell proliferation data.

- Body weight change parameters,
- Terminal body weight,

  Mean and standard deviation were calculated for each group and per time period for body weight change parameters.

  The F test was performed to compare the homogeneity of group variances.

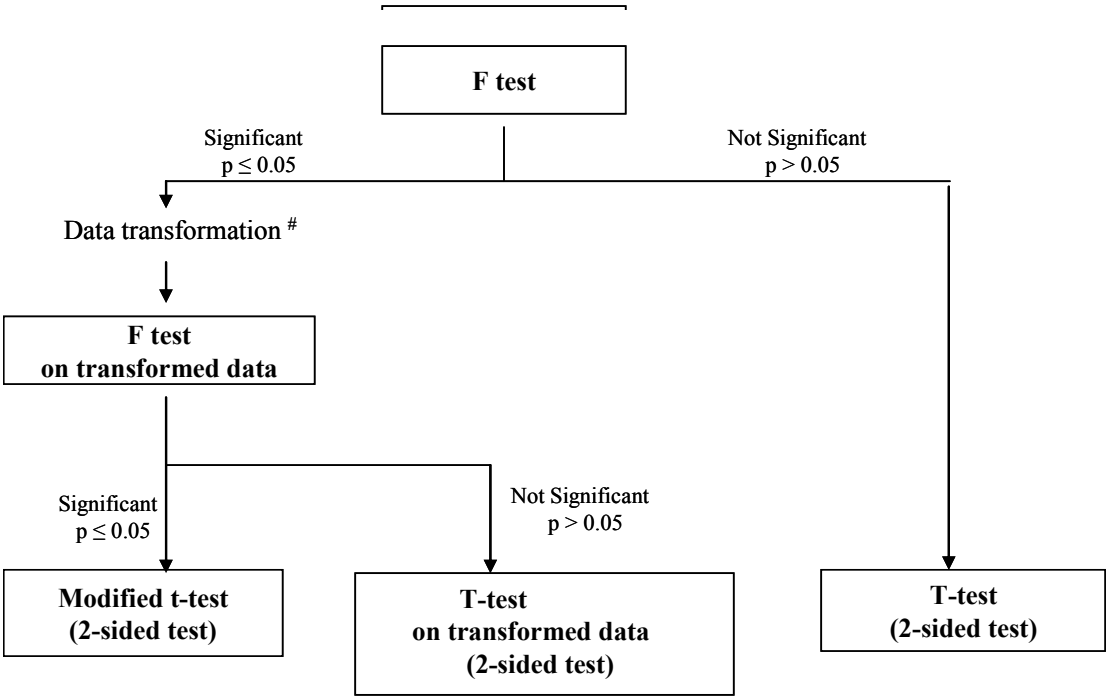
  If the F test was not significant (p>0.05), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided).

  If the F test was significant (p≤0.05), mean of the exposed group was compared to the mean of the control group using the modified t-test (2-sided).

- Body weight and average food consumption/day parameters

  Mean and standard deviation were calculated for each group and per time period for average food consumption/day parameters.

The F test was performed to compare the homogeneity of group variances.




## C. METHODS:

### 1. Observations

The animals were observed twice daily for moribundity and mortality (once daily on weekends or public holidays). Observed clinical signs were recorded at least once daily for all animals and detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility, and duration of any clinical signs were recorded.

### 2. Body weight

Each animal was weighed at least once during the acclimatization phase, on study Day 1 and then weekly during the treatment period. Additionally, animals were weighed before scheduled necropsy (terminal body weight).

### 3. Food consumption

The weight of food supplied and of that remaining at the end of the food consumption period was recorded weekly for all animals during the treatment period to calculate the mean daily food consumption. Any food spillage was noted. The weekly mean achieved dosage intake of test item in mg/kg/day for each week and for Weeks 1 to 4 was calculated for each sex using the following formula:

$$\textbf{Test item intake (mg/kg/day)} = \frac{\textbf{Dose level (ppm)} \times \textbf{Group mean food consumption (g/day)}}{\textbf{Group mean body weight (g) at the end of the food consumption period}}$$

**4. Water consumption**

Drinking water bottles containing BrdU were weighed on the first day of BrdU administration (Study Day 23). Empty water bottles were weighed at least once before scheduled sacrifice (Study Day 30).

**5. Sacrifice and pathology**

**Necropsy procedure – Organ sampling**

On Study Day 30, all animals from all groups were sacrificed by exsanguination under deep anesthesia by inhalation of Isoflurane ([redacted], France).

All animals were necropsied. The necropsy included the examination of the external surfaces, all orifices and all major organs, tissues and body cavities. Significant macroscopic abnormalities were recorded and sampled upon the decision of the Study Pathologist.
The following organs or tissues were sampled:

Thyroid gland (with trachea) Liver
Duodenum

Macroscopic findings were sampled at the discretion of the Study Pathologist.
The thyroid gland, the duodenum and the liver from each animal in all groups were preserved in 10% neutral buffered formalin.

Conventional histopathological examination

Histological samples containing 6 thyroid sections, one piece of duodenum and 2 sections of liver from all animals in all groups were embedded in paraffin wax. Histological slides, stained with hematoxylin and eosin, were prepared for these three organs from all the animals in all groups.

BrdU staining for cell proliferation measurement

For each animal in each group a section of a formalin-fixed paraffin-embedded block containing 6 thyroid samples and one sample of duodenum was prepared. The duodenum was included to serve as a positive control for staining as it has a high rate of cell proliferation. The immunohistochemical reaction included incubation with a monoclonal antibody raised against BrdU, amplification with a secondary biotinylated antibody and a streptavidin-horseradish complex, detection of the complex with diamino-benzidine (DAB) and nuclear counter staining with hematoxylin.

**6. Histopathology**

Histopathological examinations were performed on the thyroid samples from all animals in all groups. Following the initial histopathological examination, a review of representative slides was performed by a second pathologist according to standard operating procedures.

**7. Cell proliferation assessment**

The staining for BrdU and determination of the labeling index was performed on all animals showing sufficient BrdU incorporation (estimated by water consumption or duodenal BrdU labeling) to assess cell cycling in the thyroid. The labeling index, expressed as the number of

BrdU-positive thyroid follicular cells per thousand, was measured separately on multiple fields comprising of at least 1000 cells using an image analysis system (slide scanner, Hamamatsu). The mean and standard deviation were calculated for each group.

## II. RESULTS AND DISCUSSION

### A. OBSERVATIONS:

Mortality:

There were no mortalities during the course of this study.

Clinical signs:

Fluopyram: There were no treatment-related clinical signs.

Phenobarbital: All mice displayed reduced motor activity form Study Day 2 onwards.

### B. BODY WEIGHT AND BODY WEIGHT GAIN:

Fluopyram: There was no effect on body weight parameters.

Phenobarbital: Mean body weight was reduced by between 3.5% and 4.8% throughout the treatment period, the effect being statistically significant on Study Days 8 ($p \leq 0.05$), 22 ($p \leq 0.05$) and 29 ($p \leq 0.01$). This effect resulted from a mean body weight loss of 0.07 g ($p \leq 0.01$), compared with a 0.02 g mean weight gain in the controls, during the first week of treatment. Thereafter, mean body weight gain was slightly lower than in the controls during the last two weeks of treatment, resulting in an overall cumulative body weight gain of 1.0 g, compared to 2.1 g in the controls (-52%; $p \leq 0.01$), by Study Day 29.

### C. FOOD CONSUMPTION:

Fluopyram: Food consumption was unaffected by treatment throughout the dosing phase.

Phenobarbital: Food consumption was unaffected by treatment throughout the dosing phase.

### D. WATER CONSUMPTION:

Fluopyram: Mean consumption of water containing BrdU was decreased by 7.9% (not statistically significant), compared to the control group.

Phenobarbital: Mean consumption of water containing BrdU was decreased by 7.9% ($p \leq 0.05$), compared to the control group.

### E. POST MORTEM EXAMINATIONS:

**Terminal body weight**

Fluopyram: There was no change in mean terminal body weight in treated animals when compared

to the controls.

Phenobarbital: Terminal body weight was 4.4% lower than the controls and was statistically significant ($p \leq 0.01$).

**F. GROSS PATHOLOGY:**

Fluopyram: Enlarged and dark livers were noted in 14/15 and 5/15 treated animals, respectively.

Phenobarbital: Enlarged and dark livers were noted in 3/15 and 4/15 animals, respectively.

**G. MICRSCOPIC PATHOLOGY:**

Only the thyroid glands were examined.

Fluopyram: No treatment-related microscopic change was noted.

Phenobarbital: No treatment-related microscopic change was noted.

**H. CELL PROLIFERATION:**

Fluopyram: The mean BrdU labeling index was found to be approximately 1.7 fold higher ($p \leq 0.01$) than the controls.

Phenobarbital: The mean BrdU labeling index was similar to the controls.

**Table 5.5.4-94: Mean BrdU labeling index (proliferation rate/100 cells)**

| | Control | Fluopyram | Phenobarbital |
|---|---|---|---|
| **N** | **13** | **15** | **14** |
| **Mean** | 12.45 | 21.09 ** | 12.68 |
| **STD** | **6.17** | **5.78** | **3.71** |

**: $p \leq 0.01$

## III. CONCLUSION

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.

| | |
|---|---|
| **Report:** | **KIIA 5.5.4 /30; [redacted] 2013; M-449821-03; Amended: 2013-05-14** |
| Title: | Fluopyram: 28-day toxicity study for thyroid cell proliferation in the C57BL/6J male mouse |
| Report No & Document No | SA 12058<br>M-449821-03 |
| **Guidelines:** | **No specific guideline** |
| **Deviations:** | **none** |
| **GLP** | **yes** |

**Executive summary**

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 follicular cell proliferation in the thyroid revealed a 1.21, 1.40, 1.61 and 2.33 fold increase in BrdU labeling index at 150, 600, 750 and 1500 ppm, respectively, when compared to the controls, the effect being statistically significant at the three highest dose levels.

**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 dose-related increase in thyroid follicular cell proliferation, as evidenced by a 1.21, 1.40, 1.61 and 2.33 fold increase in BrdU labeling index at 150, 600, 750 and 1500 ppm, respectively, compared to the controls, was observed following dietary administration of fluopyram for at least 28 days, to the male C57BL/6J mouse. This increase in thyroid follicular cell proliferation at 1500 ppm was found to be reversible after a 28 day wash out period

## I. MATERIALS AND METHODS

### A. MATERIALS:

**1. Test Material:** Fluopyram (AE C656948)
- **Description:** Beige powder
- **Lot/Batch:** Mix-Batch: 08528/0002
- **Purity:** 94.7 %
- **CAS:** 658066-35-4
- **Stability of test compound:** Stable in rodent diet for a period covering the study duration

**2. Vehicle and/or positive control:** Vehicle for AE C656948: rodent diet
No positive control

**3. Test animals:**
- **Species**: Mouse – Male only
- **Strain:** C57BL/6J
- **Age:** 8 weeks approximately at start of treatment
- **Weight at start of dosing:** 19.4 to 23.9 g
- **Acclimation period:** 14 days
- **Diet:** Certified rodent powdered and irradiated diet A04C-10 P1 from [redacted] ([redacted], [redacted], France), *ad libitum*
- **Water:** Tap water, *ad libitum*
- **Housing:** Animals were caged individually in suspended stainless steel wire mesh cages.
- **Environmental conditions –**
  - **Temperature:** 20 – 24°C
  - **Humidity:** 40 – 70 %
  - **Air changes:** Approximately 10-15 changes per hour
  - **Photoperiod:** Alternating 12-hour light and dark cycles (7 am- 7 pm)

### B. STUDY DESIGN:

**1. In life dates:** Dosing period from May 09 to June 07, 2012. Recovery period for animals allocated to the recovery phase was from June 07 to July 04, 2012.

**2. Animal assignment and treatment**

Seven groups were dosed by dietary administration. One group consisted of control animals that received untreated diet and the remaining six groups received fluopyram at the appropriate dietary concentration (30, 75, 150, 600, 750 and 1500 ppm) at a constant level. The levels for fluopyram was set after evaluation of the results from previous studies conducted with fluopyram (SA 10241 and SA 11123) and following discussions with the US (EPA) and Canadian (PMRA) authorities. The level of 750 ppm used in the present study represents the highest level used in the mouse cancer bioassay in which an increase in thyroid tumors was observed in the males (SA 05094).

A solution of BrdU at 80 mg of BrdU/100 ml of drinking water was administered to all animals during the last week of the study.

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 control or test diet (1500 ppm fluopyram) for at least 28 days and were then allowed one month (at least 28 days) of recovery during which they were maintained on untreated control diet.

All animals were sacrificed in the morning after the last day of treatment or after the last day of the recovery phase.

**Table 5.5.4-95: Study design**

| GROUP | TEST ITEM | DOSE LEVEL | NUMBER OF ANIMALS PER GROUP | ANIMAL IDENTITY |
|---|---|---|---|---|
| MALES | | | | |
| 1 | Control | 0 ppm | 15 + 15* | T1M0546 to 0575 |
| 2 | Fluopyram | 30 ppm (5 mg/kg/day) | 15 | T2M0576 to 0590 |
| 3 | Fluopyram | 75 ppm (13 mg/kg/day) | 15 | T3M0591 to 0605 |
| 4 | Fluopyram | 150 ppm (25 mg/kg/day) | 15 | T4M0606 to 0620 |
| 5 | Fluopyram | 600 ppm (99 mg/kg/day) | 15 | T5M0621 to 0635 |
| 6 | Fluopyram | 750 ppm (124 mg/kg/day) | 15 | T6M0636 to 0650 |
| 7 | Fluopyram | 1500 ppm (247 mg/kg/day) | 15 + 15* | T7M0651 to 0680 |

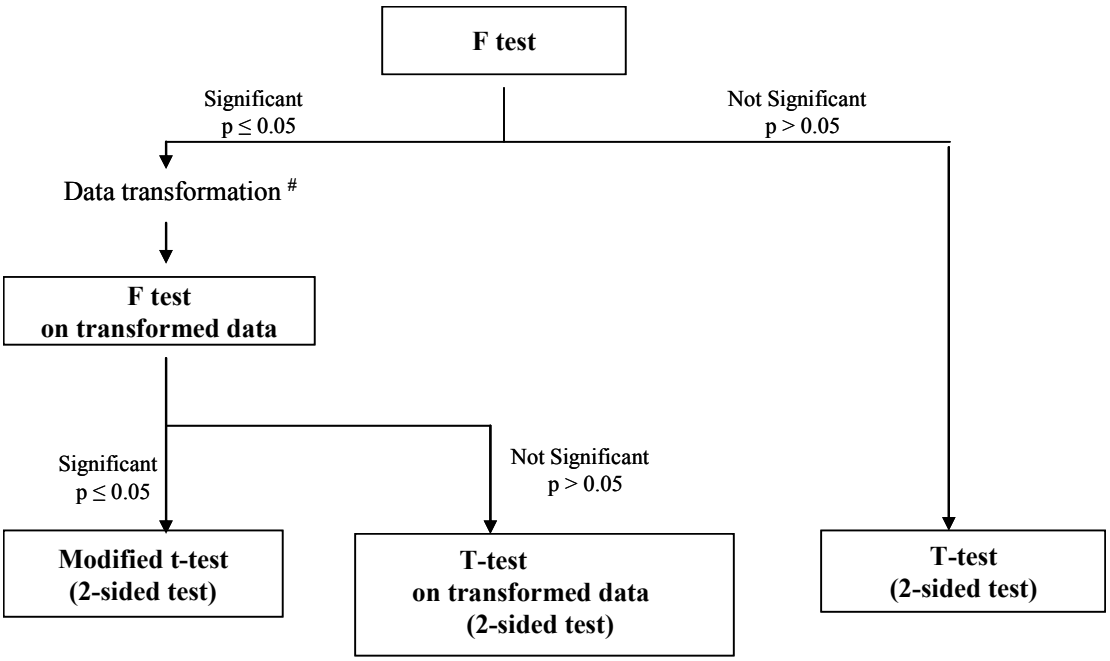
***Recovery phase**

**3. Diet preparation and analysis**

Fluopyram was incorporated into the diet to provide the required dietary concentrations.

The test formulations were stored at room temperature and issued to the animal unit in polyethylene containers. There was one formulation used in the study for each concentration and any unused diet was discarded at the end of each administration period.

The homogeneity of fluopyram in diet was verified at the lowest and highest concentrations, the mean values obtained from the homogeneity check were taken as measured concentrations. For the remaining concentrations, the dietary levels were verified for each concentration at the time of preparation. The homogeneity and concentration data for fluopyram in rodent diet were between 80 and 108% of the nominal concentration. All values were within the in-house target range of 85-115% of the nominal concentration, which the exception of one analysis at 30 ppm, which was at 80% of the nominal concentration. As this value was only slightly outside the target range and in




Variables analyzed

- Body weight parameters
- Body weight change parameters calculated according to time intervals
- Average food consumption/day parameters calculated according to time intervals
- Terminal body weight
- Thyroid cell proliferation parameters

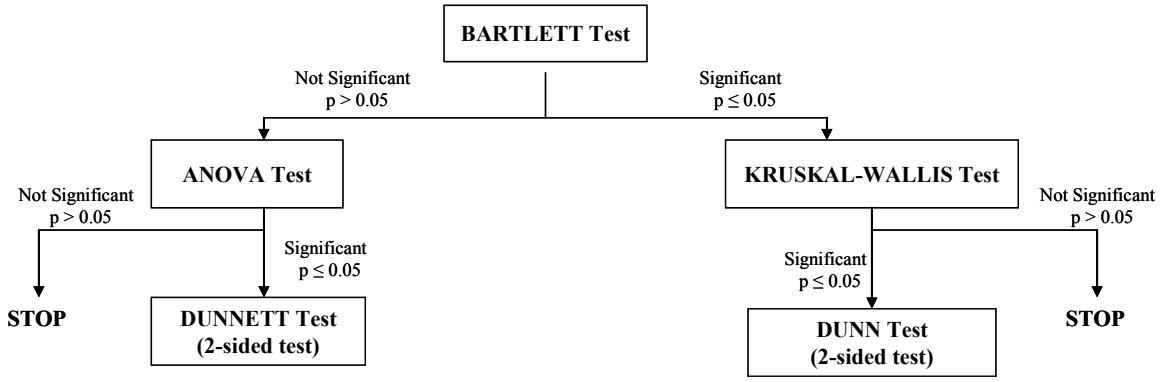
Statistical methods

Group means were compared at the 5% and 1% levels of significance.

With the exception of those used for the cell proliferation data, all statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics). SAS programs (version 9.2) were used for the cell proliferation data.

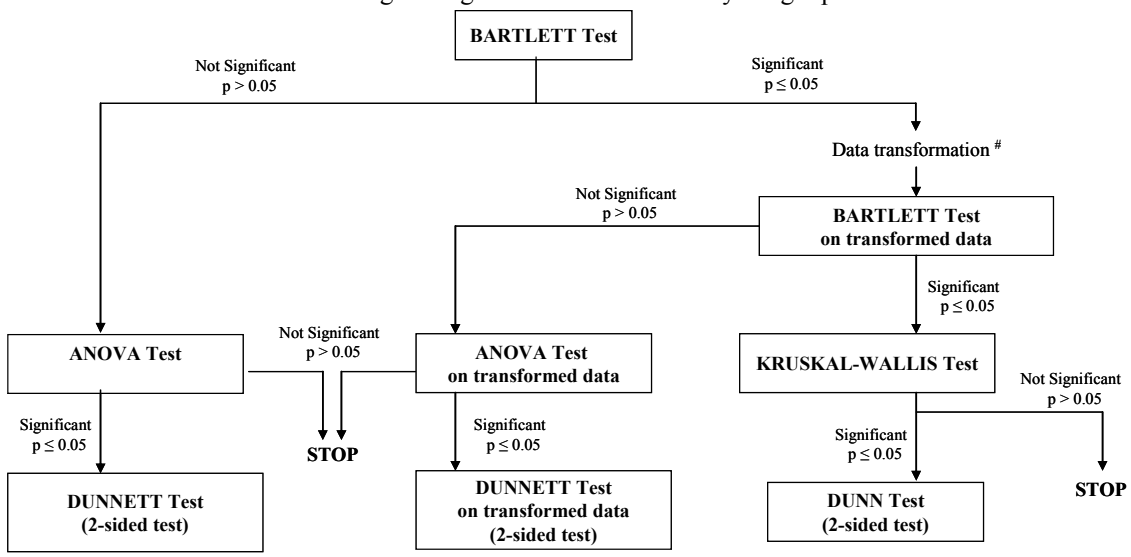
**Dosing phase fluopyram: comparison between the treated groups and the vehicle control group**

- Body weight change parameters
- Terminal body weight




- Body weight and average food consumption/day parameters

Data will be transformed using the log transformation for body weight parameters




- Thyroid follicular cell parameters

The Levene test was performed to compare the homogeneity of group variances.

If the Levene test was not significant (p>0.05), means of the exposed groups were compared to the mean of the control group using the ANOVA test.
If the ANOVA test was not significant (p>0.05), STOP.
If the ANOVA test was significant (p≤0.05), means of the exposed groups were compared to the mean of the control group using the Dunnett test (1-sided).

If the Levene test was significant (p≤0.05), data were transformed using the log transformation and means of the exposed groups were compared to the mean of the control group using the Levene test on log transformed data.
If the Levene test on log transformed data was not significant (p>0.05), means of the exposed groups were compared to the mean of the control group using the ANOVA test on log

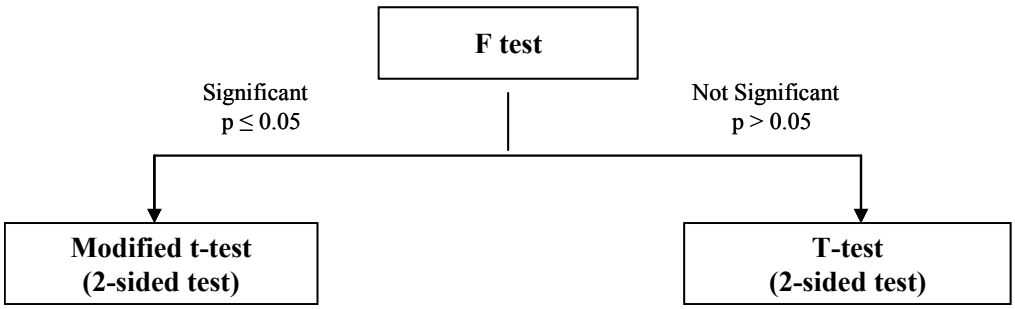
transformed data.
If the ANOVA test on log transformed data was not significant (p>0.05), STOP.
If the ANOVA test on log transformed data was significant (p≤0.05), means of the exposed groups were compared to the mean of the control group using the Dunnett test (1 sided) on log transformed data.

If the Levene test was significant (p≤0.05) even after log transformation, means of the exposed groups were compared to the mean of the control group using the Kruskal-Wallis test.
If the Kruskal-Wallis test was not significant (p>0.05), STOP.
If the Kruskal-Wallis test was significant (p≤0.05), means of the exposed groups were compared to the mean of the control group using the Dunn test (1-sided).

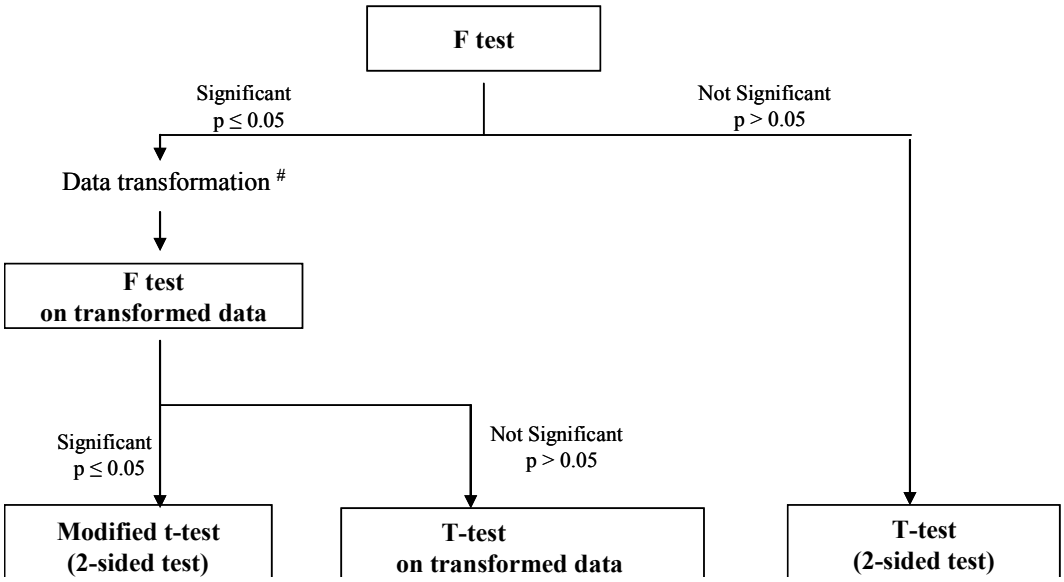
If one or more group variance(s) equal 0, means will be compared using non-parametric procedures.

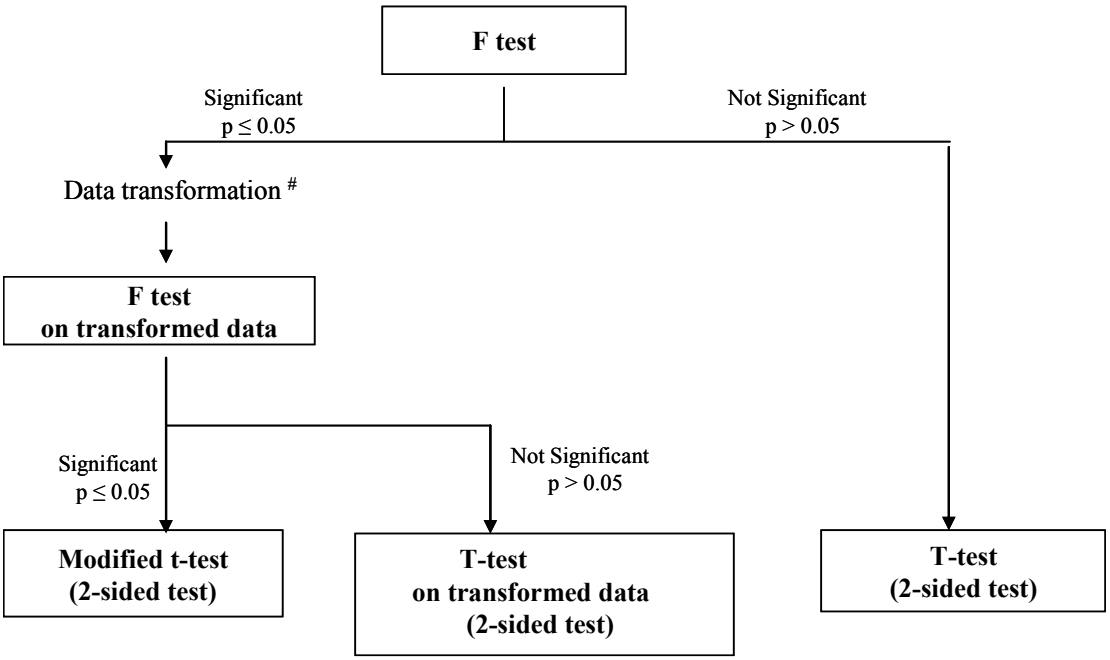
**Recovery phase fluopyram: comparison between the treated groups and the vehicle control group**

- Body weight change parameters
- Terminal body weight




- Body weight and average food consumption/day parameters




## C. METHODS:

### 1. Observations

The animals were observed twice daily for moribundity and mortality (once daily on weekends or public holidays). Observed clinical signs were recorded at least once daily for all animals and detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility, and duration of any clinical signs were recorded.

### 2. Body weight

Each animal was weighed at least once during the acclimatization phase, on study Day 1 and then weekly during the treatment period and recovery phase. Additionally, animals were weighed before scheduled necropsy (terminal body weight).

**3. Food consumption**

The weight of food supplied and of that remaining at the end of the food consumption period was recorded weekly for all animals during the treatment period to calculate the mean daily food consumption. Any food spillage was noted. The weekly mean achieved dosage intake of test item in mg/kg/day for each week and for Weeks 1 to 4 was calculated for each sex using the following formula:

$$\textbf{Test item intake (mg/kg/day)} = \frac{\textbf{Dose level (ppm)} \times \textbf{Group mean food consumption (g/day)}}{\textbf{Group mean body weight (g) at the end of the food consumption period}}$$

**4. Water consumption**

Drinking water bottles containing BrdU were weighed on the first day of BrdU administration (Study Day 23 following treatment and Study Day 22 following the recovery phase). Empty water bottles were weighed at least once before scheduled sacrifice (Study Day 30 following treatment and Study Day 29 following the recovery phase).

**5. Sacrifice and pathology**

**Necropsy procedure – Organ sampling**

On Study Days 30 and 31 following treatment or on Study Day 29 following the recovery phase, all surviving animals were sacrificed by exsanguination under deep anesthesia by inhalation of Isoflurane ([redacted], France). Animals were not diet fasted prior to sacrifices**.**

All animals were necropsied. The necropsy included the examination of the external surfaces, all orifices and all major organs, tissues and body cavities. Significant macroscopic abnormalities were recorded and sampled upon the decision of the Study Pathologist.
The following organs or tissues were sampled:

Thyroid gland (with trachea)
Duodenum

Macroscopic findings were sampled at the discretion of the Study Pathologist.
The thyroid gland, the duodenum and the liver from each animal in all groups were preserved in 10% neutral buffered formalin.

Histological preparation

Histological samples containing 6 thyroid sections and one piece of duodenum from all animals in all groups were embedded in paraffin wax. Histological slides, stained with hematoxylin and eosin, were prepared for these two organs from all the animals in all groups.

BrdU staining for cell proliferation measurement

For each animal in each group a section of a formalin-fixed paraffin-embedded block containing 6 thyroid samples and one sample of duodenum was prepared. The duodenum was included to serve as a positive control for staining as it has a high rate of cell proliferation.

The immunohistochemical reaction included incubation with a monoclonal antibody raised against BrdU, amplification with a secondary biotinylated antibody and a streptavidin-horseradish complex, detection of the complex with diamino-benzidine (DAB) and nuclear counter staining with hematoxylin.

### 6. Histopathology

Histopathological examinations were performed on the thyroid samples from all animals in all groups. Following the initial histopathological examination, a review of representative slides was performed by a second pathologist according to standard operating procedures.

### 7. Cell proliferation assessment

The staining for BrdU and determination of the labeling index was performed on all animals showing sufficient BrdU incorporation (estimated by water consumption or duodenal BrdU labeling) to assess cell cycling in the thyroid. The labeling index, expressed as the number of BrdU-positive thyroid follicular cells per thousand, was measured separately on multiple fields comprising of at least 1000 cells using an image analysis system (slide scanner, Hamamatsu). The mean and standard deviation were calculated for each group.

## II. RESULTS AND DISCUSSION

### A. OBSERVATIONS:

Mortality:

There were no treatment-related mortalities during the course of this study.

Clinical signs:

There were no treatment-related clinical signs.

### B. BODY WEIGHT AND BODY WEIGHT GAIN:

There was no treatment-related effect on body weight parameters.

### C. FOOD CONSUMPTION:

Food consumption was unaffected by treatment.

### D. WATER CONSUMPTION:

Water consumption was unaffected by treatment.

### E. POST MORTEM EXAMINATIONS:

**Terminal body weight**

Mean terminal body weight was unaffected by treatment.

## F. GROSS PATHOLOGY:

No treatment-related macroscopic findings were observed.

## G. MICRSCOPIC PATHOLOGY:

Only the thyroid glands were examined.

No treatment-related microscopic changes were noted

## H. CELL PROLIFERATION:

Dosing phase

A 1.21, 1.40, 1.61 and 2.33 fold increase in BrdU labeling index was observed at 150, 600, 750 and 1500 ppm, respectively, when compared to the control group, the effect being statistically significant at the three highest dose levels.

**Table 5.5.4-96: Mean BrdU labeling index (proliferation rate/100 cells)**

| | Control | 30 ppm | 75 ppm | 150 ppm | 600 ppm | 750 ppm | 1500 ppm |
|---|---|---|---|---|---|---|---|
| N | 14 | 15 | 15 | 15 | 15 | 15 | 15 |
| Mean | 21.55 | 17.81 | 19.51 | 26.09 | 30.11** | 34.78*** | 50.21*** |
| STD | 4.75 | 7.37 | 5.64 | 8.62 | 8.53 | 7.61 | 10.24 |

**: $p \leq 0.01$ ***: $p \leq 0.001$

Recovery phase

No change in proliferative index was noted at 1500 ppm when compared to controls.

**Table 5.5.4-97: Mean BrdU labeling index (proliferation rate/100 cells)**

| | Control | 1500 ppm |
|---|---|---|
| **N** | **14** | **14** |
| **Mean** | 17.57 | 11.56 |
| **STD** | 5.18 | 4.78 |

## III. CONCLUSION

In conclusion, a clear dose-related increase in thyroid follicular cell proliferation, as evidenced by a 1.21, 1.40, 1.61 and 2.33 fold increase in BrdU labeling index at 150, 600, 750 and 1500 ppm, respectively, compared to the controls, was observed following dietary administration of fluopyram for at least 28 days, to the male C57BL/6J mouse. This increase in thyroid follicular cell proliferation at 1500 ppm was found to be reversible after a 28 day wash out period.

| | |
|---|---|
| **Report:** | KIIA 5.5.4 /31; [redacted] 2013; M-449890-01 |
| Title: | Fluopyram: 28-day dietary study to determine potential role of the nuclear pregnane 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) |
| Report No & Document No | SA 12162<br>M-449890-01 |
| **Guidelines:** | **No specific guideline** |
| **Deviations:** | **none** |
| **GLP** | **no** |

**Executive summary**

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 [redacted] 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≤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≤0.001) as were PROD (150 fold, P≤0.001), BQ (7.9 fold, P≤0.001), T4-GT (1.9 fold, P≤0.001) and BIL-GT (2.0 fold, P≤0.001) activities.

In the pituitary gland, *Tshb* gene transcripts were up-regulated (1.7 fold, P≤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≤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≤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≤0.001) as were PROD (70 fold, P≤0.001), BQ (5.5 fold, P≤0.001), T4-GT (1.8 fold, P≤0.001) and BIL-GT (1.8 fold, P≤0.001) activities.

In the pituitary gland, *Tshb* gene transcripts were up-regulated (1.6 fold, P≤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≤0.01) whereas BQ and T4-GT activities were decreased (1.7 fold, P≤0.001 and 1.3 fold, P≤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 wild type mice.

These data, together with the increases in the glucuronidation of bilirubin and thyroxine seen in wild type 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.

# I. MATERIALS AND METHODS

## A. MATERIALS:

**1. Test Material:** Fluopyram (AE C656948)
**Description:** Beige powder
**Lot/Batch:** Mix-Batch: 08528/0002
**Purity:** 94.7 %
**CAS:** 658066-35-4
**Stability of test compound:** Stable in rodent diet for a period covering the study duration

**2. Vehicle and /or positive control:** Vehicle for AE C656948: rodent diet
No positive control

**3. Test animals:**
**Species**: Mouse – Male only
**Strain:** C57BL/6J and Pxr KO/Car KO
**Age:** 10 weeks approximately at start of treatment
**Weight at start of dosing:** 22.1 to 26.9 g wildtype mice and 20.5 to 25.0 g Pxr KO/Car KO mice
**Acclimation period:** 10 days
**Diet:** Certified rodent powdered and irradiated diet A04C-10 P1 from [redacted] ([redacted], [redacted], France), *ad libitum*
**Water:** Tap water, *ad libitum*
**Housing:** Animals were caged individually in suspended stainless steel wire mesh cages.
**Environmental conditions –**
**Temperature:** 20 – 24°C
**Humidity:** 40 – 70 %
**Air changes:** Approximately 10-15 changes per hour
**Photoperiod:** Alternating 12-hour light and dark cycles (7 am- 7 pm)

## B. STUDY DESIGN:

**1. In life dates:** Dosing period from September 24 to October 23, 2012.

**2. Animal assignment and treatment**

For both the wildtype and Pxr KO/Car KO mice, one group of control animals received untreated diet, whilst other two groups received fluopyram at the appropriate dietary concentration (750 ppm and 1500 ppm) at a constant level.

The dose levels for fluopyram were set after evaluation of the results from previous studies conducted with fluopyram (SA 10241, SA 11123) and following discussions with the US (EPA) and Canadian (PMRA) authorities. The dose level of 750 ppm is the top dose level used in the mouse cancer bioassay in which an increase in thyroid tumors was observed in the males (SA 05094). The top dose level of 750 ppm used in the mouse cancer bioassay has been shown to induce a slight but statistically significant increase in thyroid follicular cell proliferation following 28-day treatment (SA 11123).

An additional dose level (1500 ppm) in the current study was investigated to better elucidate the level of thyroid follicular cell proliferation induced after a high dose level of exposure of the test item.

A solution of BrdU at 80 mg of BrdU/100 ml of drinking water was administered to all animals during the last week of the study.

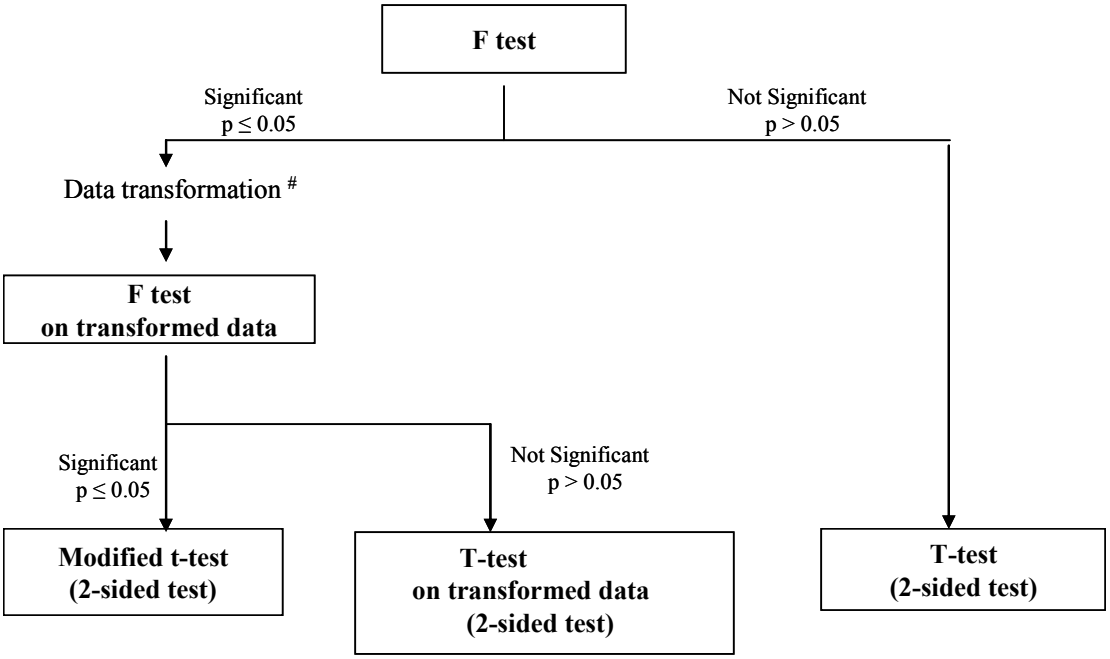
**Table 5.5.4-98: Study design**

| GROUP | TEST ITEM | DOSE LEVEL | NUMBER OF ANIMALS PER GROUP | | ANIMAL IDENTITY | | | |
|---|---|---|---|---|---|---|---|---|
| | | | C57BL/6J | Pxr KO/Car KO | C57BL/6J | | Pxr KO/Car KO | |
| | | | | | SG1 | SG2 | SG1 | SG2 |
| 1 | Control | 0 | 15 | 15 | T1M 2737 to 2743 | T1M2752 to 2759 | T1M2744 to 2751 | T1M2760 to 2766 |
| 2 | Fluopyram | 750 ppm | 15 | 15 | T2M2767 to 2774 | T2M2782 to 2788 | T2M2775 to 2781 | T2M2789 to 2796 |
| 3 | | 1500 ppm | 15 | 15 | T3M2797 to 2803 | T3M2812 to 2819 | T3M2804 to 2811 | T3M2820 to 2826 |

SG: Subgroup

**3. Diet preparation and analysis**

Fluopyram was incorporated into the diet to provide the required dietary concentration. The method of preparation was documented in the study file. The test item formulation was stored at room temperature and issued to the animal unit in polyethylene containers. There was one




Variables analyzed

- Body weight parameters
- Body weight change parameters calculated according to time intervals
- Average food consumption/day parameters calculated according to time intervals
- Terminal body weight, absolute and relative organ weights parameters

– Total cytochrome P-450 content and liver enzyme activities
– Cell proliferation parameters
– Quantity of gene transcripts

Statistical methods

Mean and standard deviation were calculated for each group.
P-450 content and enzymatic activities were not statistically analyzed in Pristima; only descriptive statistics are presented.
Group means were compared at least at the 5% levels of significance.
Statistical analyses were carried out using Pristima, version 6.3.2 build 17, Xybion Corp.

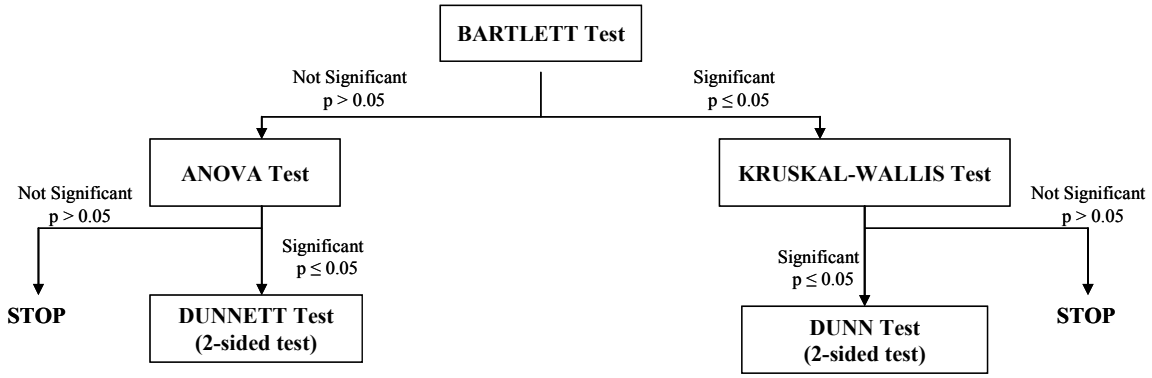
- - Total cytochrome P-450 content and liver enzyme activities

Statistical comparisons between fluopyram treated mice and their respective control groups were performed for all numerical data sets using a 2-tailed Student's t-test.

- - Body weight change parameters,
  - Terminal body weight, absolute and relative organ weight parameters,

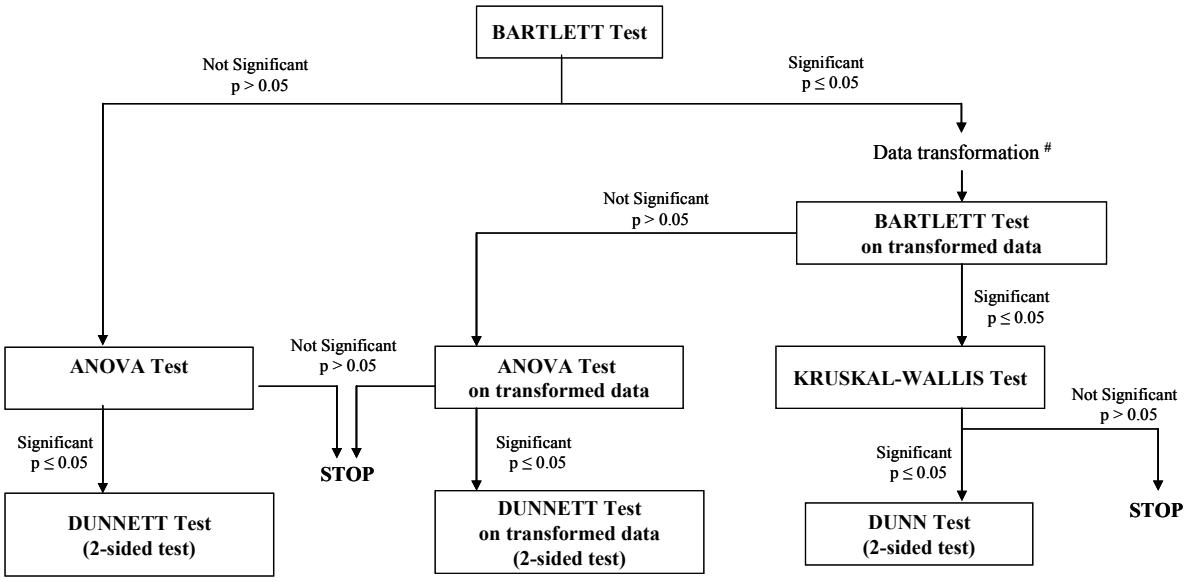
Mean and standard deviation were calculated for each group and per time period for body weight change parameters.

– Terminal body weight




- - Body weight and average food consumption/day parameters
  - Quantity of gene transcripts

Mean and standard deviation were calculated for each group and per time period for average food consumption/day parameters.




Data were transformed using the log transformation for body weight, food consumption and gene transcript parameters.

– Thyroid follicular cell proliferation parameters

The Levene test was performed to compare the homogeneity of group variances.

If the Levene test was not significant (p>0.05), means of the exposed groups were compared to the mean of the control group using the ANOVA test.
If the ANOVA test was not significant (p>0.05), STOP.
If the ANOVA test was significant (p≤0.05), means of the exposed groups were compared to the mean of the control group using the Dunnett test (1-sided).

If the Levene test was significant (p≤0.05), data were transformed using the log transformation and means of the exposed groups were compared to the mean of the control group using the Levene test on log transformed data.
If the Levene test on log transformed data was not significant (p>0.05), means of the exposed groups were compared to the mean of the control group using the ANOVA test on log transformed data.
If the ANOVA test on log transformed data was not significant (p>0.05), STOP.
If the ANOVA test on log transformed data was significant (p≤0.05), means of the exposed groups were compared to the mean of the control group using the Dunnett test (1 sided) on log transformed data.

If the Levene test was significant (p≤0.05) even after log transformation, means of the exposed groups were compared to the mean of the control group using the Kruskal-Wallis test.
If the Kruskal-Wallis test was not significant (p>0.05), STOP.
If the Kruskal-Wallis test was significant (p≤0.05), means of the exposed groups were compared to the mean of the control group using the Dunn test (1-sided).

If one or more group variance(s) equal 0, means will be compared using non-parametric procedures.

## C. METHODS:

### 1. Observations

The animals were observed twice daily for moribundity and mortality (once daily on weekends or public holidays). Observed clinical signs were recorded at least once daily for all animals and detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility, and duration of any clinical signs were recorded.

### 2. Body weight

Each animal was weighed at least once during the acclimatization phase, on study Day 1 and then weekly during the treatment period and recovery phase. Additionally, animals were weighed before scheduled necropsy (terminal body weight).

### 3. Food consumption

The weight of food supplied and of that remaining at the end of the food consumption period was recorded weekly for all animals during the treatment period to calculate the mean daily food consumption. Any food spillage was noted. The weekly mean achieved dosage intake in mg/kg/day for each week and for Weeks 1 to 4 was calculated for each sex using the following formula:

$$\textbf{Test item intake (mg/kg/day)} = \frac{\textbf{Dose level (ppm)} \times \textbf{Group mean food consumption (g/day)}}{\textbf{Group mean body weight (g) at the end of the food consumption period}}$$

### 4. Water consumption

Drinking water bottles containing BrdU were weighed on the first day of BrdU administration (Study Day 23). Empty water bottles were weighed at least once before scheduled sacrifice (Study Day 30).

### 5. Clinical Chemistry

On Study Day 30, blood samples were taken from all animals in all groups by puncture of the abdominal aorta. Animals were anesthetized by inhalation of Isoflurane ([redacted], France). Blood was collected on heparin for possible further analysis.

### 6. Sacrifice and pathology

**Necropsy procedure – Organ sampling**

On Study Day 30, all surviving animals were sacrificed by exsanguination under deep anesthesia by inhalation of Isoflurane ([redacted], France). Animals were not diet fasted prior to sacrifices.

All animals were necropsied. The necropsy included the examination of all major organs, tissues and body cavities. Significant macroscopic abnormalities were recorded and sampled upon decision of the Study Pathologist.

At final sacrifice the following organs were weighed.

- Brain
- Liver

Tissue collection and histotechnology

The following organs were sampled.

- Duodenum
- Liver
- Pituitary gland
- Thyroid gland

For each animal, the thyroid gland, two central sections taken from the median and left lobes of the liver and a portion of the duodenum were preserved 48 hours in 10% neutral buffered formalin.

Six sections of the thyroid gland, one piece of duodenum and two pieces of liver from all animals were processed and embedded in paraffin wax. Histological sections were prepared for all animals in all groups and stained with hematoxylin and eosin.

An immunohistochemical staining demonstrating the incorporation of BrdU and the determination of the labeling index was performed to assess thyroid follicular cell cycling on all study animals. A section from a formalin-fixed paraffin-embedded block containing 6 thyroid sections and one duodenum sample was prepared. The immunohistochemical reaction included incubation with a monoclonal antibody raised against BrdU, amplification with a secondary biotinylated antibody and a streptavidin-horseradish peroxidase complex, detection of the complex with the chromogen diamino-benzidine (DAB) and nuclear counterstaining with hematoxylin.

For each group, one piece of both the median and left lobes of the liver plus the pituitary gland from each animal (both strains) were collected and flash frozen in liquid nitrogen. These samples were stored at approximately -74°C + 10°C until used for qPCR investigations.
The remaining portion of the liver from all animals were collected and flash frozen in liquid nitrogen prior to storage at approximately -70°C until shipped to [redacted] for microsomal preparations.

Histopathology and cell proliferation assessment

Histopathological examinations were performed on thyroid gland and liver for all animals. Following the initial histopathological examination, a review of representative slides was performed by a second pathologist ([redacted]) according to standard operating procedures.

A digital image of the whole slide containing the thyroid gland was acquired using the Nanozoomer 2.0 series (Hamamatsu). The labeling index, expressed as the number of BrdU-positive thyroid follicular cells per thousand, was measured on multiples fields by counting in total at least 1000 cells. The mean and standard deviation were calculated for each group.

HEPATOTOXICITY TESTING

At final necropsy, the remaining portions of the liver from all animals were collected and sent to [redacted] for liver subcellular fractionation. The liver was processed according to [redacted] LMS Cent-001. Microsomes were isolated and stored at approximately -70°C until required for total cytochrome P-450 content, specific cytochrome P-450 isoenzyme profile and glucoronidation assessments to check the hepatotoxic potential of the test item.

Total cytochrome P-450 content

Total cytochrome P-450 content of the liver microsomal fractions was determined according to LMS Spec-0002 ([redacted], 1964).

10.2 Protein determination

The protein concentration of the liver microsomes was determined in aqueous solutions using a modification of the method of Lowry et al., (1951) and bovine serum albumin standards, according to LMS Spec-001. Results were maintained in the study file.

10.3 Cytochrome P-450 activity assays

Microsomal pentoxyresorufin-O-depentylation (PROD) was used as a marker for Cyp2b activity, and was measured according to LMS Fluor-0002, and EQ-001 (Burke et al 1985).
Cyp3a11 activity was measured as the O-debenzylation of benzyloxyquinoline (BQ) according to LMS Fluor-0003, and EQ-001.

10.4 Thyroxine (T4) glucuronidation activity

Mouse liver microsomes were incubated with 125I-thyroxine and the formation of T4-glucuronide was determined by HPLC with radioflow detection.

10.5 Bilirubin glucuronidation activity

Bilirubin glucuronidation activity was assessed in mouse liver microsomes. Full details of the method used and the results were maintained in the Study File.

11 - QUANTITATIVE PCR ANALYSIS

11.1 Total RNA purification

Total cytoplasmic RNA was isolated from the pituitary gland of all individual control and treated animals using RNeasy Mini kits (Qiagen). RNA quality controls were performed based on the ribosomal RNA electrophoretic profiles using a Bioanalyser (Agilent Technologies).

11.2 Quantitative PCR

An appropriate quantity of total RNA were used for Reverse transcription (RT) using a High Capacity cDNA Archive kit (Applied Biosystems). The assay was performed in duplicate using Taqman assays (Assay on demand, Applied Biosystems), diluted first strand cDNA, FastStart Universal Probe Master Mix (Roche) on an ABI prism 7900 HT machine (Applied Biosystems). β-actin was used as the reference gene for the quantitative calculations.

The list of Taqman assays used was as follows:

| Gene family | Isoform | Refset ID | Taqman assay ID (Applied Biosystems) |
|---|---|---|---|
| Thyroid Stimulating Hormone beta subunit | *Tshb* | NM_009432.1 | Mm03990915-g1 |
| Beta actin | Actb | NM_007393.1 | Mm00607939_s1 |

Beta-actin (Actb) was selected as reference gene for the quantitative calculations of transcripts. The relative quantity (RQ) value of each test transcript was calculated using the following formula:

$\Delta\Delta Ct$ = (Cttest – CtB2m) treated - (Cttest - CtB2m) control

RQ = $2^{-\Delta\Delta Ct}$

where Ct is the threshold cycle at which PCR amplification started to be significantly different from the background signal. Each RQ value obtained for a given gene was normalized by dividing by the RQ value obtained for the control animal T1M2737 (C57BL/6J mice) or T1M2744 (Pxr KO/Car KO mice).

## II. RESULTS AND DISCUSSION

### A. OBSERVATIONS:

Mortality:

There were no treatment-related mortalities during the course of this study.

Clinical signs:

C57BL/6J mice:
No clinical signs were observed throughout the study.

Pxr KO/Car KO mice:
One control mouse had hair loss on both hind limbs on Study Days 29 and 30, whilst one mouse (T3M2825) from the 1500 ppm treatment group was noted to have a swollen penis on Study Days 29 and 30. These clinical signs were considered to be incidental.

### B. BODY WEIGHT AND BODY WEIGHT GAIN:

C57BL/6J mice:
Body weight parameters were unaffected by treatment throughout the study.

Pxr KO/Car KO mice:
At 750 ppm, mean body weight gain was higher than in the controls during the first week of treatment, resulting in a cumulative body weight gain of 0.35 g, compared to a cumulative body weight loss of 0.06 g in the controls ($p \leq 0.05$), by Study Day 8. This difference was considered incidental in the absence of an overall effect on cumulative body weight change at 750 ppm and since body weight parameters were unaffected by treatment at the higher dose level of 1500 ppm.

## C. FOOD CONSUMPTION AND ACHIEVED DOSAGES:

C57BL/6J mice:

Food consumption parameters were unaffected by treatment throughout the study compared to the controls.

Pxr KO/Car KO mice:

At 750 ppm, mean food consumption was decreased by between -9% ($p \leq 0.01$; Study Day 15) and -4% (not statistically significant; Study Day 8) compared to the controls. In the absence of an effect on mean food consumption at the higher dose level of 1500 ppm, the decreased food consumption noted at 750 ppm was considered to be incidental.

ACHIEVED DOSAGES

The mean achieved dietary intake of fluopyram expressed in mg/kg/day received by the animals during the study was as follows:

**Table 5.5.4-99: Mean achieved dietary intake of fluopyram**

| Mean achieved dietary intake of fluopyram (Weeks 1 – 4) | | |
|---|---|---|
| Diet concentration (ppm) | C57BL/6J (mg/kg/day) | Pxr KO/Car KO (mg/kg/day) |
| 750 | 125 | 130 |
| 1500 | 256 | 247 |

## D. WATER CONSUMPTION:

Water consumption was unaffected by treatment.

## E. POST MORTEM EXAMINATIONS:

**Terminal body weight and organ weights**

At terminal sacrifice, there were no changes in mean terminal body weights between control and wild-type (C57BL/6J) mouse groups or between control and Knock-Out (Pxr KO/Car KO) mouse groups.

In wild type animals, at 1500 and 750 ppm, mean absolute and relative liver weights were statistically significantly higher when compared to controls. These changes were dose-related and were considered to be treatment-related.

**Table 5.5.4-100: Mean liver weight changes; Wild-Type Mice**

| MEAN LIVER WEIGHT ±SD AT SCHEDULED SACRIFICE, WILD-TYPE GROUPS (% CHANGE WHEN COMPARED TO CONTROLS) | | | |
|---|---|---|---|
| Sex | Males | | |
| Fluopyram dose level (ppm) | 0 | 750 | 1500 |
| Mean absolute liver weight (g) | 1.277<br>±0.06859 | 1.8035**<br>±0.15861<br>(+41%) | 2.115***<br>±0.19106<br>(+66%) |
| Mean liver to body weight ratio (%) | 4.9285<br>±0.18549 | 6.8482**<br>±0.44559<br>(+39%) | 7.9883***<br>±0.48285<br>(+62%) |
| Mean liver to brain weight ratio (%) | 288.9877<br>±17.75730 | 409.6459**<br>±35.06164<br>(+42%) | 480.1812***<br>±43.73024<br>(+66%) |

**P≤0.01; ***P≤0.001

In Knock-Out animals, at 1500 and 750 ppm, mean absolute and relative liver weights were statistically significantly higher when compared to controls. These changes were dose-related and were considered to be treatment-related.

**Table 5.5.4-101: Mean liver weight changes; Knock-Out Mice**

| MEAN LIVER WEIGHT ±SD AT SCHEDULED SACRIFICE, KNOCK-OUT GROUPS (% CHANGE WHEN COMPARED TO CONTROLS) | | | |
|---|---|---|---|
| Sex | Males | | |
| Fluopyram dose level (ppm) | 0 | 750 | 1500 |
| Mean absolute liver weight (g) | 1.2945<br>±0.08045 | 1.4008**<br>±0.07952<br>(+8%) | 1.4307**<br>±0.07729<br>(+11%) |
| Mean liver to body weight ratio (%) | 5.3119<br>±0.24617 | 5.7075**<br>±0.27193<br>(+7%) | 5.7970**<br>±0.23142<br>(+9%) |
| Mean liver to brain weight ratio (%) | 297.0051<br>±15.48663 | 315.5953*<br>±26.98886<br>(+6%) | 336.8503**<br>±18.1761<br>(+13%) |

*P≤0.05; **P≤0.01; ***P≤0.001

## B. GROSS PATHOLOGY:

C57BL/6J mice:

Enlarged livers were noted at 1500 and 750 ppm. These changes were associated with microscopic hepatocellular hypertrophy and were considered to be treatment-related.

**Table 5.5.4-102: Macroscopic changes in the Liver; Wild-Type Mice**

| INCIDENCE OF MACROSCOPIC CHANGES IN THE LIVER, SCHEDULED SACRIFICE, WILD-TYPE GROUPS | | | |
|---|---|---|---|
| Sex | Males | | |
| Fluopyram dose level (ppm) | 0 | 750 | 1500 |
| Enlarged | 0/15 | 7/15 | 14/15 |

Pxr KO/Car KO mice:
No macroscopic change was noted.

## C. MICRSCOPIC PATHOLOGY:

C57BL/6J mice:
At 1500 ppm, hepatocellular hypertrophy, hepatocellular single cell necrosis, increased number of mitoses and interstitial mixed cell infiltrate were noted.
At 750 ppm, hepatocellular hypertrophy, hepatocellular single cell necrosis and interstitial mixed cell infiltrate were noted.

**Table 5.5.4-103: Microscopic changes in the Liver; Wild-Type Mice**

| INCIDENCE OF MICROSCOPIC CHANGES IN THE LIVER, SCHEDULED SACRIFICE, WILD-TYPE GROUPS | | | |
|---|---|---|---|
| Sex | Males | | |
| Fluopyram dose level (ppm) | 0 | 750 | 1500 |
| Number of examined animals | 15 | 15 | 15 |
| **Hepatocellular hypertrophy: centrilobular to panlobular** | | | |
| Minimal | 0 | 0 | 2 |
| Slight | 0 | 14 | 6 |
| Moderate | 0 | 1 | 7 |
| Total | 0 | 15 | 15 |
| **Hepatocellular single cell necrosis: focal** | | | |
| Minimal | 0 | 5 | 9 |
| Slight | 0 | 0 | 1 |
| Total | 0 | 5 | 10 |
| **Increased number of mitoses** | | | |
| Present | 0 | 0 | 3 |
| **Interstitial mixed cell infiltrate: focal** | | | |
| Minimal | 5 | 9 | 11 |
| Slight | 1 | 0 | 0 |
| Total | 6 | 9 | 11 |

Pxr KO/Car KO mice:
No relevant microscopic change was noted at 1500 or 750 ppm.

## D. CELL PROLIFERATION:

C57BL/6J mice:
Higher proliferative indexes were noted (2.6-fold at 1500 ppm and 1.8-fold at 750 ppm) when compared to controls.

**Table 5.5.4-104: Mean BrdU labeling index (proliferation rate/100 cells); Wild-Type Mice**

| Thyroid gland proliferation index | | | |
|---|---|---|---|
| Fluopyram dose level (ppm) | 0 | 750 | 1500 |
| N | 15 | 15 | 15 |
| Mean | 14.28 | 26.08*** | 36.61*** |
| STD | 3.96 | 7.16 | 10.27 |

***P≤0.001

Pxr KO/Car KO mice:

No change was noted between control and treated groups.

**Table 5.5.4-105: Mean BrdU labeling index (proliferation rate/100 cells); Knock-Out Mice**

| Thyroid gland proliferation index | | | |
|---|---|---|---|
| Fluopyram dose level (ppm) | 0 | 750 | 1500 |
| N | 15 | 15 | 15 |
| Mean | 10.05 | 9.91 | 8.27 |
| STD | 3.88 | 4.06 | 3.38 |

## E. HEPATOXICITY TESTING:

C57BL/6J mice:
The total cytochrome P450 content of the liver microsomal fractions from male C57BL/6J mice administered fluopyram at 1500 ppm and 750 ppm for 28 days was increased 3.7- and 3.6-fold, respectively, over the concurrent control.

Administration of fluopyram at 1500 ppm and 750 ppm for 28 days increased hepatic microsomal PROD (a marker for Cyp2b) 151- and 70-fold in male C57BL/6J mice, respectively, over the concurrent control.

28 day exposure to fluopyram at 1500 ppm and 750 ppm increased microsomal BQ (a marker for Cyp3a11) activity by 7.9- and 5.5-fold concurrent controls in male C57BL/6J mice, respectively.

Following 28 days exposure to fluopyram at 1500 ppm and 750 ppm, microsomal thyroxine glucuronosyl transferase (T4-GT) activity was increased by 1.9- and 1.8-fold concurrent controls in male C57BL/6J mice, respectively.

Bilirubin glucuronidation in male C57BL/6J mice administered fluopyram at 1500 ppm and 750 ppm for 28 days was increased 2.0-, and 1.8-fold, respectively, over the concurrent control.

**Table 5.5.4-106: Results of Hepatotoxicity testing; Wild-Type Mice**

| Parameter | Control 0 ppm | Fluopyram 750 ppm | Fluopyram 1500 ppm |
|---|---|---|---|
| C57BL/6J | | | |
| Total P450 nmol/mg protein | 0.34 ± 0.22 (100.0 ± 63.5) | 1.24 ± 0.16*** [a] (363.3 ± 47.2) | 1.25 ± 0.18*** (367.3 ± 51.3) |
| PROD pmols resorufin formed/min/mg protein | 2.01 ± 0.20 (100.0 ± 10.0) | 140.21 ± 15.11*** [a] (6991.0 ± 753.5) | 302.14 ± 84.76*** (15065.3 ± 4226.4) |
| BQ nmols 7-OH quinoline formed /min/mg protein | 2.77 ± 0.34 (100.0 ± 12.3) | 15.20 ± 1.89*** [a] (549.3 68.3) | 21.94 ± 1.83*** (792.8 ± 66.1) |
| T4-GT pmol T4-glucoronide formed/min/mg protein | 0.58 ± 0.17 (100.0 ± 29.9) | 1.06 ± 0.17*** [a] (183.8 ± 29.2) | 1.09 ± 0.25*** (189.5 ± 43.6) |
| BIL-GT nmol bilirubin-glucuronide formed/min/mg protein | 0.73 ± 0.11 (100.0 ± 14.4) | 1.30 ± 0.22*** [a] (177.4 ± 30.0) | 1.43 ± 0.42*** (195.6 ± 57.6) |

[a] Values are Mean ± SD. Values in parenthesis are mean % control ± SD. n = 15 per group; [a] n = 14
A Students' t-test (2-sided) was performed on the results; *statistically different from control p<0.05; **p<0.01; *** p<0.001.

Pxr KO/ Car KO mice:
Fluopyram had no effect on the total microsomal P450 content in the Pxr KO/Car KO mice.

Both at 1500 and 750 ppm, Pxr KO/Car KO mice showed little induction of PROD in comparison to wild type animals, even if the effect was statistically significant (1.4-fold).

Administration of fluopyram at 1500 ppm and 750 ppm to male Pxr KO/Car KO mice for 28 days decreased BQ activity to 1.7-fold and 1.5-fold of concurrent control values, respectively.

Administration of fluopyram to male Pxr KO/Car KO mice for 28 days had no effect on T4-GT at 750 ppm, and decreased T4-GT activity to 1.3-fold of concurrent control values at 1500 ppm.

Fluopyram had no effect on bilirubin glucuronidation in the Pxr KO/Car KO mice.

**Table 5.5.4-107: Results of Hepatotoxicity testing; Knock-Out Mice**

| Parameter | Control 0 ppm | Fluopyram 750 ppm | Fluopyram 1500 ppm |
|---|---|---|---|
| Pxr KO/Car KO | | | |
| Total P450 nmol/mg protein | 0.24 ± 0.09 (100.0 ± 36.5) | 0.27 ± 0.12 (112.3 ± 49.7) | 0.27 ± 0.12 (111.0 ± 47.5) |
| PROD pmols resorufin formed/min/mg protein | 2.27 ± 0.30 (100.0 ± 13.1) | 3.20 ± 0.81*** (141.3 ± 35.6) | 3.24 ± 1.16** (142.9 ± 51.0) |
| BQ nmols 7-OH quinoline formed /min/mg protein | 3.51 ± 0.39 (100.0 ± 11.0) | 2.40 ± 0.40*** (68.5 ± 11.3) | 2.09 ± 0.35*** (59.4 ± 10.1) |
| T4-GT pmol T4-glucoronide formed/min/mg protein | 0.57 ± 0.19 (100.0 ± 34.0) | 0.66 ± 0.17 (115.9 ± 29.9) | 0.43 ± 0.15* (75.3 ± 26.7) |
| BIL-GT nmol bilirubin-glucuronide formed/min/mg protein | 0.70 ± 0.24 [a] (100.0 ± 34.1) | 0.66 ± 0.24 [a] (94.5 ± 34.1) | 0.61 ± 0.28 (86.5 ± 39.4) |

[a] Values are Mean ± SD. Values in parenthesis are mean % control ± SD. n = 15 per group; [a] n = 14
A Students' t-test (2-sided) was performed on the results; *statistically different from control p<0.05; **p<0.01; *** p<0.001.

## F. Q-PCR ANALYSIS:

C57BL/6J mice:

*Tshb* gene transcripts were up-regulated in a dose related manner (1.7-fold, p≤0.001 and 1.6-fold, p≤0.01 and at 1500 and 750 ppm, respectively) in the pituitary gland of fluopyram treated male mice when compared with controls.

**Table 5.4.4-108: *Tshb* gene transcript levels; Wild-Type Mice**

| | Mean Relative Quantity ± standard deviation of gene transcripts (% change compared to control mean values) | | |
|---|---|---|---|
| Gene transcripts | Control | Fluopyram 750 ppm | Fluopyram 1500 ppm |
| *Tshb* | 1.23 ± 0.289 | 1.92** ± 0.306 (+56%) | 2.05*** ± 0.586 (+67%) |

**P≤0.01; ***P≤0.001

Pxr KO/Car KO mice:

*Tshb* gene transcripts were slightly down-regulated (1.2-fold, p≤0.05) at 1500 ppm and very slightly down-regulated (1.1-fold, not statistically significant) at 750 ppm in the pituitary gland of male mice treated with fluopyram when compared with controls. Due to the low amplitude of these deregulations, these changes were considered as non-biologically relevant.

**Table 5.4.4-109: *Tshb* gene transcript levels; Wild-Type Mice**

| | **Mean Relative Quantity ± standard deviation of gene transcripts (% change compared to control mean values)** | | |
|---|---|---|---|
| Gene transcripts | Control | Fluopyram 750 ppm | Fluopyram 1500 ppm |
| *Tshb* | 1.25 ± 0.264 | 1.14 ± 0.177 | 1.04* ± 0.165 (-17%) |

*P≤0.05

## III. 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 wild type mice.

These data, together with the increases in the glucuronidation of bilirubin and thyroxine seen in wild type 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.

| | |
|---|---|
| **Report:** | **KIIA 5.5.4 /34; [redacted] 2013; M-451628-01** |
| Title: | Fluopyram: Assessment of pentoxyresorufin-*o*-depentylation and benzyloxyquinoline-*o*-debenzylation in 50 liver microsomal samples |
| Report No & Document No | CXR1284<br>M-451628-01 |
| **Guidelines:** | **No specific guideline** |
| **Deviations:** | **none** |
| **GLP** | **no** |

**Executive summary**


- 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 where in-life phase of the study was conducted under the Study number SA 11105, Documentation No. M-428031-02, [redacted] (2012): Fluopyram – Mechanistic 28-day toxicity study in the mouse by dietary administration (hepatotoxicity and thyroid hormone investigations).
- 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.

# I. MATERIALS AND METHODS

## A. MATERIALS:

**1. Test Material:** Fluopyram (AE C656948)
**Description:** Beige powder
**Lot/Batch:** Mix-Batch: 08528/0002
**Purity:** 94.7 %
**CAS:** 658066-35-4
**Stability of test compound:** Stable in rodent diet for a period covering the study duration

**2. Vehicle and /or positive control:** Vehicle for AE C656948: rodent diet
Phenobarbital (positive control)
Vehicle for Phenobarbital: methylcellulose
**Positive control:** Phenobarbital
**Description:** White crystalline powder
**Lot/Batch:** Lot No. 09050075
**Purity:** 100 %
**CAS:** 50-06-6
**Stability of test compound:** Stable in 0.5% aqueous solution of methylcellulose for a period covering the study duration

**3. Test animals:**
**Species**: Mouse – Male only
**Strain:** C57BL/6J
**Age:** 8 weeks approximately at start of treatment
**Weight at start of dosing:** 18.1 to 24.7 g
**Acclimation period:** 13 to 15 days
**Diet:** Certified rodent powdered and irradiated diet A04C-10 P1 from [redacted] ([redacted], [redacted] France), *ad libitum*
**Water:** Tap water, *ad libitum*
**Housing:** Animals were caged individually in suspended stainless steel wire mesh cages.
**Environmental conditions –**
**Temperature:** 20 – 24°C
**Humidity:** 40 – 70 %
**Air changes:** Approximately 10-15 changes per hour
**Photoperiod:** Alternating 12-hour light and dark cycles (7 am- 7 pm)

## B. STUDY DESIGN:

**1. In life dates:** Dosing period from May 24 to June 24, recovery period from June 22 to July 26, 2011
**2. Microsomal analysis dates:** March 14 to 18, 2013

**3. Animal assignment and treatment**

Seven groups of male mice were dosed for at least 28 days with either control diet or the appropriate compound by the appropriate route of administration.

Six groups were dosed by dietary administration. One group consisted of control animals that received untreated diet and the remaining five groups received fluopyram at the appropriate dietaryconcentration (30, 75, 150, 600 and 750 ppm) at a constant level. The dose levels for fluopyramwere set after evaluation of the results from previous studies and following discussions with the US (EPA) and Canadian (PMRA) authorities. The top dose level of 750 ppm used in the present studyrepresents the top dose level used in the mouse cancer bioassay in which thyroid tumors wereobserved in the males.

The seventh group was dosed by oral gavage with 80 mg/kg/day phenobarbital suspended in 0.5%aqueous methylcellulose 400 using a dosing volume of 5 ml/kg bodyweight the first week of treatment and then using a dosing volume of 10 ml /kg bodyweight for the remaining treatment period. The volume administered to each mouse was based on the most recent recorded body weight. A dose level of 80 mg/kg/day was chosen for phenobarbital as this dose was used in previous mouse mechanistic studies conducted with phenobarbital, where thyroid changes were detected.

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 control or test diet (750 ppm fluopyram) or were orally dosed (80 mg/kg/day phenobarbital) for at least 28 days and were then allowed one month (at least 28 days) of recovery during which they were maintained on untreated control diet.

All animals were sacrificed in the morning after the last day of treatment or after the last day of the recovery phase.

**Table 5.5.4-110: Study design**

| GROUP | TEST SUBSTANCE | DOSE LEVEL | NUMBER OF ANIMALS PER GROUP | ANIMAL IDENTITY |
|---|---|---|---|---|
| MALES | | | | |
| 1 | Control | 0 | 15 + 15* | T1M1885 to 1914 |
| 2 | Fluopyram | 30 ppm (5 mg/kg/day) | 15 | T2M1915 to 1929 |
| 3 | | 75 ppm (13 mg/kg/day) | 15 | T3M1930 to 1944 |
| 4 | | 150 ppm (25 mg/kg/day) | 15 | T4M1945 to 1959 |
| 5 | | 600 ppm (102 mg/kg/day) | 15 | T5M1960 to 1974 |
| 6 | | 750 ppm (128 mg/kg/day) | 15 + 15* | T6M1975 to 2004 |
| 7 | Phenobarbital | 80 mg/kg/day | 15 + 15* | T7M2005 to 2034 |

**4. Statistics**

Statistical comparisons between treated microsomes and their control group were undertaken for all numerical data sets using a 2-tailed Student's t-test.

## C. METHODS:

### 1.1 Microsomal samples

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).

### 1.2. Pentoxyresorufin-*O*-depentylation (PROD)

The activity of Cyp2b in microsomal samples was determined spectrofluorometrically by the formation of resorufin from pentoxyresorufin, as described by Burke *et al* (1985) Biochem. Pharmacol. 34, 18. 3337-3345, according to LMS Fluor-002.

### 1.3. Benzyloxyquinoline-*O*-debenzylation (BQ)

The activity of Cyp3a in microsomal samples was determined spectrofluorometrically by the formation of 7-hydroxyquinoline from benzyloxyquinoline, as described by GENTEST HTS technical bulletin, according to LMS Fluor-005.

## II. RESULTS AND DISCUSSION

### B. Pentoxyresorufin-*O*-depentylation:

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.

**Table 5.5.4-111: Pentoxyresorufin-*O*-Depentylation**

| Treatment, 28 day exposure | PROD (pmol resorufin formed/min/mg protein) |
|---|---|
| Control | 4.65 ± 1.12 (100.0 ± 24.2)[a] |
| Fluopyram 30ppm | 67.22 ± 6.95*** (1447.1 ± 149.5) |
| Fluopyram 75ppm | 156.89 ± 46.37*** (3377.5 ± 998.3) [a] |
| Fluopyram 150ppm | 170.99 ± 29.09*** (3680.8 ± 626.2) |
| Fluopyram 600ppm | 201.51 ± 45.02*** (4338.0 ± 969.1) |
| Fluopyram 750ppm | 219.06 ± 31.31*** (4715.8 ± 674.0) |
| Phenobarbital 80mg/kg/day | 151.87 ± 48.84*** (3269.3 ± 1051.4) |
| **Treatment, 28 day recovery** | |
| Control | 4.63 ± 0.82 (100.0 ± 17.7) |
| Fluopyram 750ppm | 5.27 ± 0.75 (113.9 ± 16.1) [a] |
| Phenobarbital 80mg/kg/day | 5.33 ± 0.31 (115.1 ± 6.6) |

[a] Values are Mean ± SD. Values in parenthesis are mean % control ± SD. n = 5 per group; [a] n = 4 per group. A Student's t-test (2-sided) was performed on the results; *statistically different from control p<0.05; ** p<0.01; ***p<0.001.

**Figure 5.5.4-12: Pentoxyresorufin-*O*-Depentylation, 28 Day Exposure**

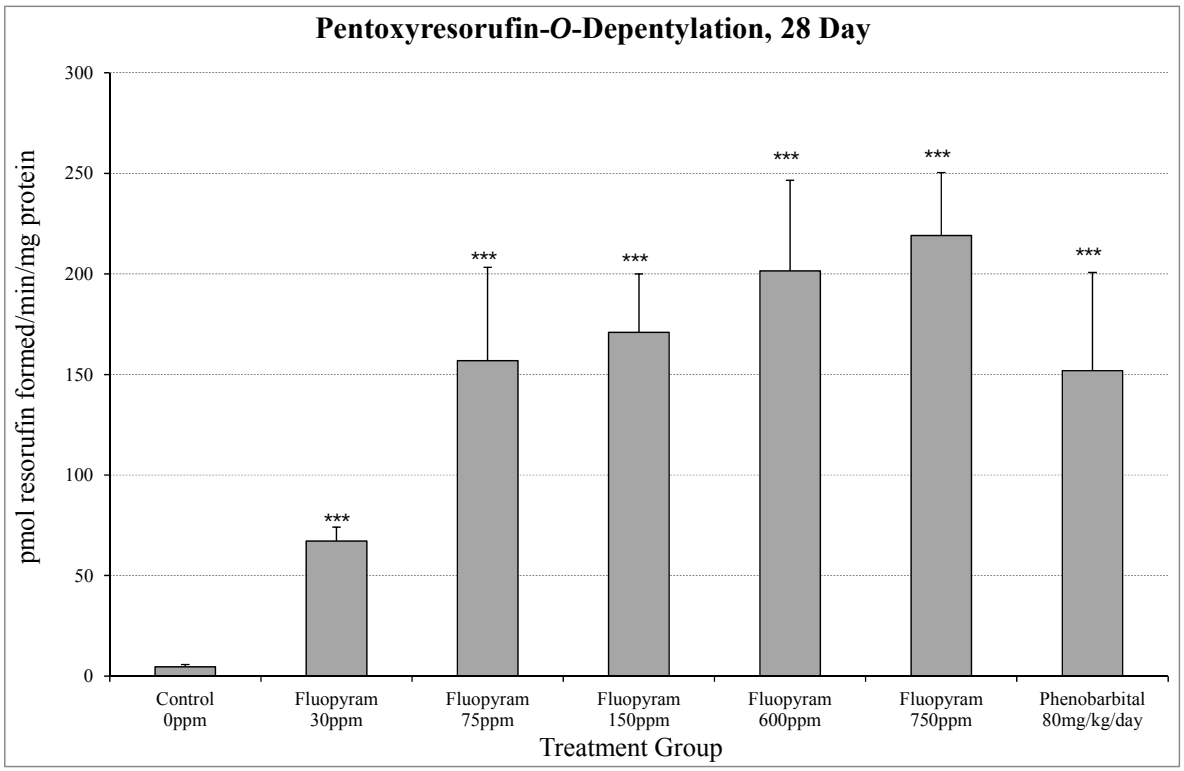
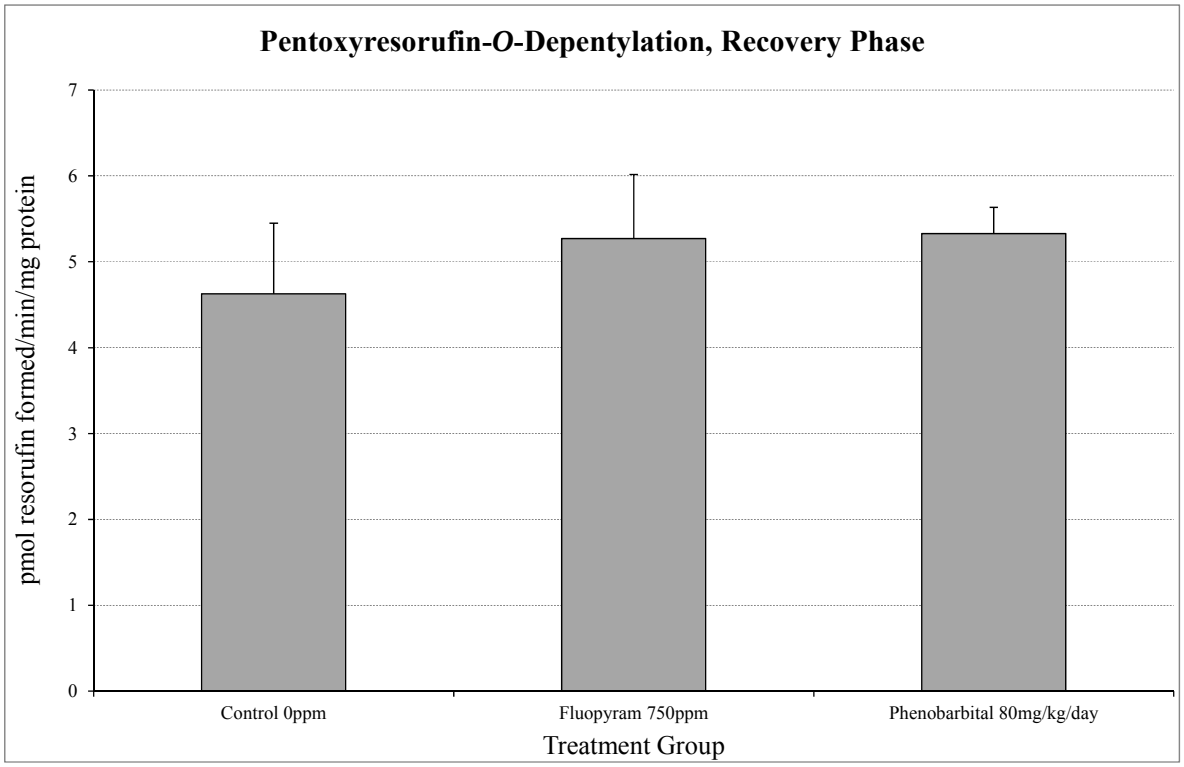



**Figure 5.5.4-13**: **Pentoxyresorufin-*O*-Depentylation, 28 Day Recovery**



## 2. Benzyloxyquinoline-*O*-Debenzylation

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.

**Table 5.5.4-112: Benzyloxyquinoline-*O*-Debenzylation**

| Treatment, 28 day exposure | BQ (nmol 7-Hydroxyquinoline formed/min/mg protein) |
|---|---|
| Control | 7.59 ± 0.67 (100.0 ± 8.8)[a] |
| Fluopyram 30ppm | 10.68 ± 1.92* (140.7 ± 25.3) |
| Fluopyram 75ppm | 16.71 ± 2.61*** (220.2 ± 34.4) [a] |
| Fluopyram 150ppm | 21.98 ± 1.76*** (289.7 ± 23.2) |
| Fluopyram 600ppm | 39.20 ± 7.49*** (516.6 ± 98.6) |
| Fluopyram 750ppm | 47.24 ± 3.85*** (622.6 ± 50.8) |
| Phenobarbital 80mg/kg/day | 23.00 ± 5.24*** (303.1 ± 69.1) |
| **Treatment, 28 day recovery** | |
| Control | 6.93 ± 1.01 (100.0 ± 14.5) |
| Fluopyram 750ppm | 6.68 ± 1.27 (96.4 ± 18.4) [a] |
| Phenobarbital 80mg/kg/day | 6.45 ± 1.22 (93.1 ± 17.7) |

[a] Values are Mean ± SD. Values in parenthesis are mean % control ± SD. n = 5 per group; [a] n = 4 per group. A Student's t-test (2-sided) was performed on the results; *statistically different from control p<0.05; ** p<0.01; ***p<0.001.

**Figure 5.5.4-14**: **Benzyloxyquinoline-*O*-Debenzylation, 28 Day Exposure**

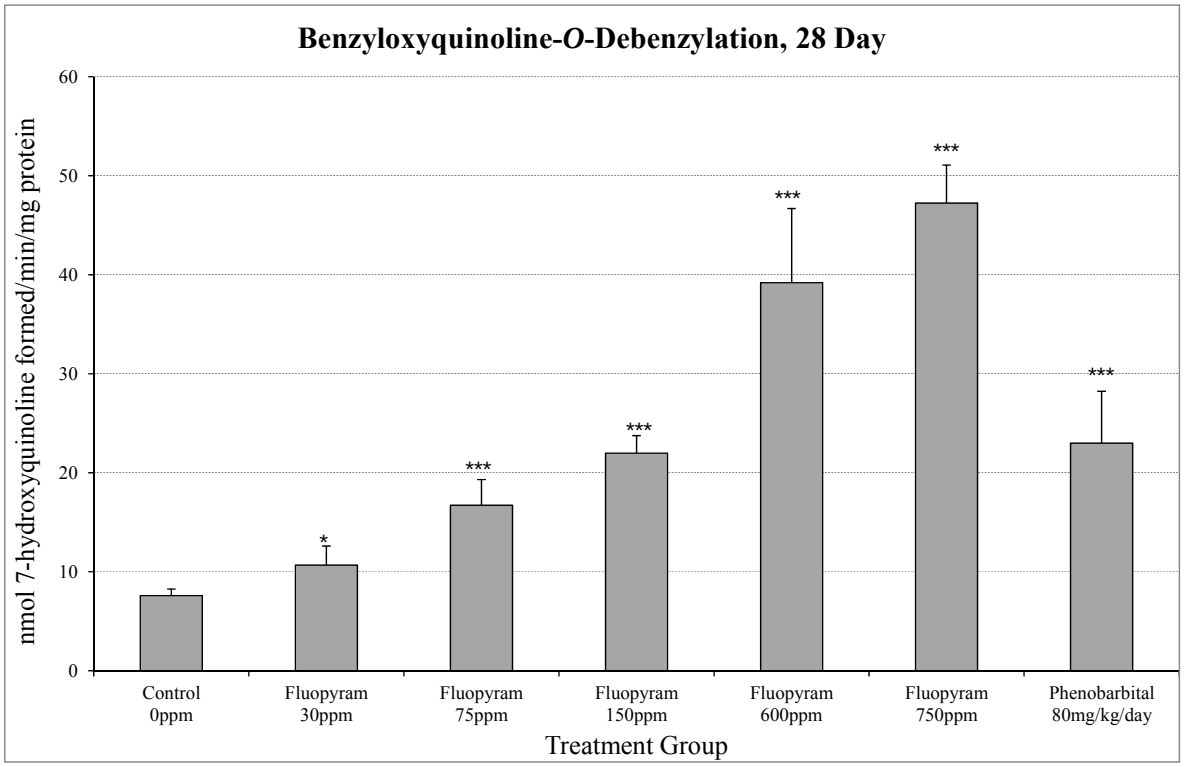
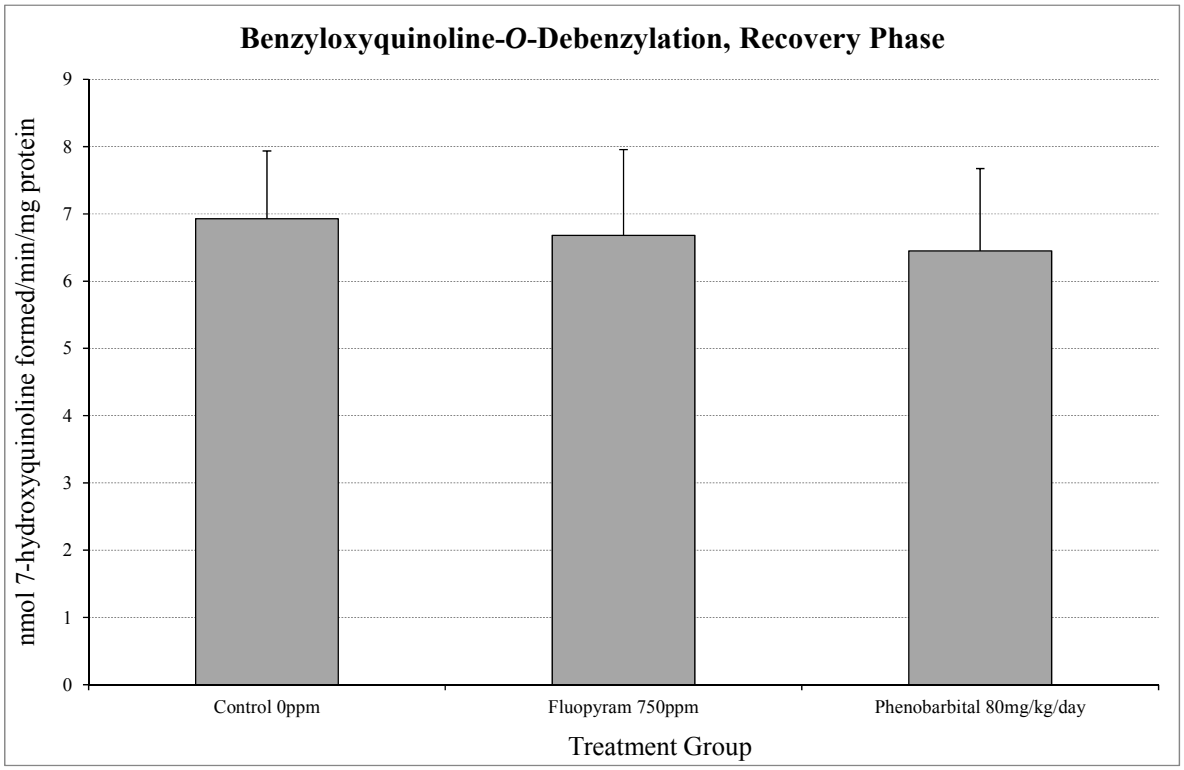



**Figure 5.5.4-15: Benzyloxyquinoline-*O*-Debenzylation, 28 Day Recovery**



## III. CONCLUSION

- 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.