



REGULATORY TOXICOLOGY

POSITION PAPER

Subject :

Penflufen

**Position paper on the proposed
classification with H351**

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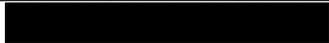
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1. Abstract

Penflufen is currently in the commenting period of the classification process by ECHA. The Rapporteur Member State (UK Competent Authority) proposed to classify penflufen as “suspected of causing cancer” Carc 2; H351 based on the presence of:

- small increases in the incidence of hepatocellular adenoma in female rats, and increased incidence of liver carcinoma in male mice (top and mid doses), which exceeded the concurrent and historical control incidence rates.
- very small increased incidences of tubulostromal adenoma at the top dose in female rats only,
- very small increased incidences of astrocytoma in male rat only at the top dose,
- very small increased incidences of histiocytic sarcoma in male rat only at top dose,

The RMS concluded that the increased tumours frequencies were slight, only just outside control ranges and they could have arisen by chance. A case could be made for no classification, on the basis of a lack of relevance to humans. However, the relevance to humans cannot be dismissed for all the tumours types and the small increases above background levels make it difficult to conclude that they were incidental.

In the present document, the summarized data show that following:

- A long term dietary administration of penflufen to male and female C57/Bl6 mice, a slightly higher incidence of adenoma and carcinoma in the liver was found in all doses in males, when compared to controls. However, these neoplastic findings were found without any dose effect relationship over a quite large range of dose levels. They were not associated with an increased incidence of pre-neoplastic changes. Overall, the incidences of those tumours were only marginally outside the historical control range for this strain of mice and this laboratory. Therefore, these liver tumours were considered not to be treatment-related. In females there was no increased incidence of hepatocellular tumour.
- A long term dietary administration of penflufen to male and female Wistar rats, numerically higher incidences of neoplastic lesions were observed in the liver. The hepatocellular adenoma observed in female rats following a 2-year treatment period with penflufen was considered to be subsequent to a phenobarbital-like mechanism of action which is a well known mechanism of action specific to the rodent and of no relevance to humans.

- A long term dietary administration of penflufen to male and female Wistar rats, numerical higher incidences of neoplastic lesions were observed in ovaries, hematopoietic system and brain in some treated dose groups, generally the high dose group. Given that these incidences were similar to internal and/or external historical control data, that these neoplastic lesions were generally not dose-related and that penflufen is devoid of any genotoxic potential, it was concluded that the neoplastic lesions found in the ovaries, hematopoietic system and brain were not related to treatment with penflufen.

Therefore, the weight of evidence of all existing information on the toxicological profile of penflufen and on the occurrence of spontaneous tumour, lead to the conclusion that tumours (benign and malignants) observed are unlikely related to the administration of the penflufen, and that penflufen is not considered to present a carcinogenic risk to humans.

2.Introduction

The general toxicity profile of penflufen is characterized by very low acute toxicity, no mutagenicity, no reproduction and developmental toxicity.

Penflufen represents a new class of chemistry (alkylamide) and belongs to the FRAC Fungicide Group 7 (Carboxamides) also known as SDHI fungicides. Its biochemical mode of action has been shown to inhibit the enzyme succinate dehydrogenase (complex II - SDH inhibitor) within the fungal mitochondrial respiratory chain, thus blocking electron transport. At the fungal cell level, penflufen has an effect on several stages of the life cycle of fungi. It has shown inhibition of spore germination, germ tube elongation and mycelium growth.

It is being developed as a seed treatment, providing a high level of protection to seedlings against seed-borne fungal diseases. Penflufen penetrates into the germinating seeds and shows a locosystemic distribution in young plants through the xylem, allowing a high level of protection of the developing seedling.

3.Astrocytoma

Two carcinogenicity studies were carried out in rodents.

In the C57BL/6J mice study (BCS 2009, M-357859-01-1), groups of 60 male and 60 female were fed diet containing 0, 100, 1000 or 6000 ppm of penflufen for 52 weeks. After 52 weeks, 10 males and 10 females from each group allocated to the chronic phase of the study were necropsied at the scheduled interim sacrifice. The remaining 50 animals/sex/group, allocated to the carcinogenicity phase of the study, continued treatment until the scheduled final sacrifice of the study after at least 78 weeks of treatment. The mean intake of penflufen over 18 months was calculated to be 0, 14.3, 146 and 880 mg/kg/day in males and 0, 18.4, 182 and 1101 mg/kg/day in females at 0, 100, 1000 and 6000 ppm, respectively. In this study, no increased incidences of brain tumours were observed.

In the Wistar rat study (BCS 2009, M-357848-01-1), groups of 80 animals per sex were fed diet containing 0, 100, 2000 and 7000 ppm of penflufen corresponding to 0, 4.0, 79 and 288 mg/kg/day in males and 0, 5.6, 113 and 399 mg/kg/day in females over 24-month treatment period. The reversibility of any effects observed after one year was assessed following a recovery of three months on untreated diet. Groups of eighty male and eighty female rats per group were used (10 animals/sex/group designated for the interim sacrifice after 52 weeks, 10 animals/sex/group designated for the recovery group sacrifice after 65 weeks and 60 animals/sex/group designated for the final sacrifice after 104 weeks). At the scheduled chronic, reversibility and carcinogenicity phase sacrifices, selected organs were weighed and designated tissues sampled and examined microscopically. In this study, a higher incidence of brain astrocytoma (3/60, 5%) was present in males at 7000 ppm (288 mg/kg/day) only (Table 1).

Table 1: Incidence of microscopic changes in the brain (male only)

Doses	0	100	2000	7000
Number of animals	60	60	60	60
Astrocytoma	1	0	0	3
Incidences	1.60%	0%	0%	5%
	Study incidence range 0-2/55 (0-3.7%)			

This incidence is slightly above our historical control data (3.7%) but is lower than the 8% in the

literature (Krinke, 2000). No dose-response was observed.

There was no increased incidence of brain tumours in female rats, although toxicokinetic data showed that penflufen bioavailability following the oral administration was higher in female, indeed the elimination is faster for males than for females (BCS 2009, M-1824534/7). In addition, the concentration of penflufen in female brain is twice those observed in male brain after administration of 2-5 mg/kg of penflufen (BCS 2009a&b, M-1811491/5). Therefore, if penflufen were a neurocarcinogen, the incidence of astrocytomas should have been increased also in females.

Brain astrocytomas belong to the neuroectodermal tumours and are commonly grouped together as gliomas including astrocytoma, oligodendroglioma and ependyma. Cellular differentiation in astrocytoma is complex and includes protoplasmic, fibrillary, gemistocytic or pilocytic tumoral astrocytes. Mixed patterns can be seen histologically, diagnosed as mixed glioma which combines both tumoral astrocytes and oligodendrocytes. This was not the case in our study.

The incidence of rat brain tumours increases with advancing age, with generally a greater prevalence in males, but it can occur spontaneously in quite young rats, even before one year's old (Son W, 2010). In our study, these tumours appear in unscheduled animals only (with no concomitant increase in terminal sacrifice animals).

The above arguments, together with the information that no brain tumours were seen in the mouse carcinogenicity, and that penflufen was not a genotoxic corroborate the fact that the small increase incidence of astrocytoma observed in rat study was incidental and not treatment related.

4. Tubulostromal adenoma

Two carcinogenicity studies were carried out in rodents.

In the mouse carcinogenicity study (BCS 2009, M-357859-01-1), no ovary findings were reported (up to 6000 ppm – 880/1101 mg/kg/day M&F).

In the rat carcinogenicity study (BCS 2009, M-357848-01-1), there was a slight increase of tubulostromal adenoma and tubulostromal hyperplasia incidences in female rats at 7000 ppm (288 mg/kg/day). The incidence of tubulostromal adenoma was slightly higher than the historical control data while the incidence of tubulostromal hyperplasia was within our internal historical control data and clearly lower than the highest value (11.9% versus 25%). 5/7 hyperplasia were recorded as minimal severity, only one marked as in the control, and no high severity grade (Table 2). The slight increase of incidence in adenoma was not associated with any increased number of adenoma per animals where each animal had only one tumour, whereas in control and low dose 2 animals have two tubulostromal adenomas each.

Table 2: Incidence and severity of microscopic changes in the ovary

Doses	0	100	2000	7000
Number of animals	60	60	60	60
Tubulostromal adenocarcinoma	0	1	1	0
Incidences	0%	1.7%	1.7%	0%
	Study incidence max 0%			
Tubulostromal adenoma	2	1	1	7
Incidences	3.3%	1.7%	1.7%	11.9%
	Study incidence max 6.7%			
Tubulostromal hyperplasia focal				
Minimal	2	0	0	5
Slight	0	2	0	0
Moderate	0	2	0	1
Marked	1	0	1	1
Incidences	3	4	1	7
	5.0%	6.6%	1.7%	11.9%
	Study incidence max 25%			

The tubulostromal tumours are specific to rodents and have no similar counterpart in humans, who mainly develop epithelial tumours (Cannistra, 2004; Greaves, 2012, [REDACTED], 2010).

In rodents, tubulostromal hyperplasias or tumours are intraparenchymatous or exophytic and well delimited, mixing both tubular structures and stromal cells. Tubulostromal adenocarcinoma, tubulostromal adenoma and tubulostromal hyperplasia have the same epithelial cellular origin.

Therefore the lack of evidence of an increased incidence of tubulostromal hyperplasia and of a transition from hyperplasia to adenoma, the lack of animals with multiple adenomas like in control or low dose group are strong arguments for stating that the slight increase of adenoma in the top dose group is not treatment related.

5.Histiocytic sarcoma

Two carcinogenicity studies were carried out in rodents.

In the mouse carcinogenicity study (BCS 2009, M-357859-01-1), no histiocytic sarcoma was observed (up to 6000 ppm – 880/1101 mg/kg/day M&F).

In the rat carcinogenicity study (BCS 2009, M-357848-01-1), an increased incidence of histiocytic sarcoma was noted in males only, just above the historical control data at the top dose (Table 3). No dose-response pattern or positive trend was evident with the incidence in the low and mid-dose groups despite a 20-fold difference in dose (100 to 2000 ppm). In addition, when considering only terminal sacrifice animals, there is clearly no-dose relationship.

Table 3: Incidence of histiocytic sarcoma (male only)

Doses	0	100	2000	7000
Number of animals	60	60	60	60
Histiocytic sarcoma	0	3	3	5
Incidences	0%	5.0%	5.0%	8.3% (0.01<p≤0.05)
	Internal HCD incidence max 6.7% RITA HCD 6% male and 5% female			
Number of animals (terminal sacrifice)	19	24	21	24
Histiocytic sarcoma	0	3	0	3
Incidences	0%	5.0%	0.0%	5.0%

Moreover, in the female, histiocytic sarcoma were observed only in the control group at 5% (3/60) and none in the treated group even at the top dose whereas the top dose is 399 mg/kg/day in female compared to male 288 mg/kg/day.

This neoplasia was not associated with any toxic or preneoplastic effects in this chronic study (up to 107 weeks) in a consistent, temporal or specific fashion.

No mechanism of action is known for induction of histiocytic sarcomas. To date in rodents, histiocytic sarcomas have been linked to exposure to mutagenic compounds (e.g. tetrafluoroethylene). Thus in

the absence of genotoxicity there is no apparent mechanism of action for induction of histiocytic sarcomas.

Since penflufen is not genotoxic and histiocytic sarcoma is not a sex-dependent tumour and there is no clear dose-response relationship, histiocytic sarcoma of the hematopoietic system in this study was considered not treatment-related.

6. Hepatic adenoma/carcinoma

Two carcinogenicity studies were performed on rodents.

In the mouse carcinogenicity study (BCS 2009, M-357859-01-1), at 6000 ppm, enlarged liver was found in both males and females (Table 4).

Table 4: Mean liver weights changes at terminal sacrifice (% change when compared to controls) in mice

Sex	Males				Females			
penflufen dose level (ppm)	0	100	1000	6000	0	100	1000	6000
Terminal BW (g)	26.4	26.9	26.3	26.0	23.8	23.6	23.7	23.5
Mean absolute liver weight (g)	1.18	1.18 (0%)	1.20 (+2%)	1.40** (+19%)	1.27	1.28 (+1%)	1.32 (+4%)	1.56** (+23%)
Mean liver to body weight ratio (%)	4.483	4.387 (-2%)	4.571 (+2%)	5.387** (+20%)	5.318	5.396 (+1%)	5.559 (+5%)	6.601** (+24%)
Mean liver to brain weight ratio (%)	260.223	258.884 (-1%)	264.743 (+2%)	310.881** (+19%)	270.814	271.587 (0%)	280.300 (+4%)	335.626** (+24%)

** : Statistically different ($p \leq 0.01$) from the control.

These liver changes were associated with an increased incidence and severity of diffuse centrilobular hepatocellular hypertrophy in both sexes at all dose levels (Table 5). These changes were found to be dose-related. At 6000 ppm, a higher incidence and severity of diffuse mainly periportal hepatocellular macrovacuolation was noted in females.

Table 5: Incidence and severity of microscopic changes in the liver in mice

Sex	Male				Female			
Penflufen dose level (ppm)	0	100	1000	6000	0	100	1000	6000
Number of animals examined	48	49	49	48	50	50	50	50
Centrilobular hepatocellular hypertrophy: diffuse								
Minimal	0	9	17	2	0	2	4	20
Slight	0	3	9	15	0	1	1	10
Moderate	0	1	3	29	0	0	0	1
Total	0	13**	29**	46**	0	3	5*	31**
Hepatocellular vacuolation: diffuse								
Minimal	8	10	7	13	16	7	14	12
Slight	2	2	4	6	20	24	16	18
Moderate	0	0	1	0	2	9	14	14
Total	10	12	12	19*	38	40	44	44
Hepatocellular macrovacuolation: mainly periportal : diffuse								
Minimal	0	0	1	1	11	8	7	9
Slight	0	0	0	0	3	3	0	22
Moderate	0	0	0	0	0	0	0	10
Total	0	0	1	1	14	11	7	41**

*: Statistically different ($p \leq 0.05$) from the control

** : Statistically different ($p \leq 0.01$) from the control

A slightly higher incidence of adenoma and carcinoma in the liver was found in all doses in males, when compared to controls (Table 6). However, these neoplastic findings were found without any dose effect relationship over a quite large range of dose levels. They were not associated with an increased incidence of pre-neoplastic changes. Overall, the incidences of those tumours were only marginally outside the historical control range for this strain of mice and this laboratory. Therefore, these liver tumours were considered not to be treatment-related. In females there was no increased incidence of hepatocellular tumours.

Table 6: Incidence of tumors in the liver - all animals - 18-month carcinogenicity phase in mice

Sex	Male				Female			
Penflufen dose level (ppm)	0	100	1000	6000	0	100	1000	6000
Number of animals examined	48	49	49	48	50	50	50	50
Hepatocellular carcinoma	1	1	3	3	0	0	0	1
Hepatocellular adenoma	1	5	1	4	1	0	1	0
Total	2	5 ^a	4	5 ^b	1	0	1	1

^a one animal with both adenoma and carcinoma

^b two animals with both adenoma and carcinoma

To conclude, the dietary administration of penflufen over an 18-month period of the C57BL/6J mouse did not present any carcinogenic potential up to the highest dose level tested of 6000 ppm (equating approximately to 880 and 1101 mg/kg body weight/day in males and females, respectively). Treatment-related increased liver weights associated with histopathological findings were observed in the liver in both sexes at 6000 ppm, associated to follicular cell hyperplasia in female only. Treatment-related histological findings were also observed in the liver of male and female mice at 1000 and 100 ppm, but they were considered not adverse according to the low severity of the changes and the absence of any effect on liver weights.

The combined chronic/carcinogenicity study in Wistar rat showed at 7000 ppm and to a lesser extent at 2000 ppm, treatment-related macroscopic findings consisted of enlarged liver and dark liver in both sexes (BCS 2009, M-357848-01-1). At the microscopic examination, in the liver, a higher incidence of centrilobular to panlobular hepatocellular hypertrophy and of centrilobular hepatocellular macrovacuolation was recorded (Table 7). In addition, in females only, a higher incidence of hepatocellular brown pigments was noted. The findings in the liver were considered to represent a non-adverse, adaptive and reversible response to the treatment secondary to increased hepatic metabolism. The liver microscopic findings were mostly reversible as liver hypertrophy was not observed after the 3-month recovery.

There was a dose-related increased incidence of hepatocellular hypertrophy in males and females at 2000 and 7000 ppm. A low number of hepatocellular neoplasms (adenoma and/or carcinoma) were noted in most groups, including control males (Table 7). At 7000 and 2000 ppm, higher incidences of liver adenoma and eosinophilic foci of alteration were observed in females. Although there was a higher incidence of carcinoma plus adenoma in the 2000 ppm dose group, this incidence was driven by the number of adenomas with little or no contribution from the single carcinoma observed only in this group. The higher incidence of adenoma was only statistically significant at 2000 ppm. While a slight numerical increase in the incidence of adenoma was present at 7000 ppm, the increase was not statistically significant as compared to the control group.

Liver enzyme induction leading to hepatocellular hypertrophy generally correlates with the degree of hepatocellular proliferative response (Allen, 2004). Proliferative hepatocellular lesions are well described in the liver of the rat in the published literature (Greaves, 1984, Goodman, 1994). The most common proliferative lesions in the liver include non-neoplastic proliferative changes (e.g. eosinophilic foci) and the neoplastic changes (adenoma and carcinoma). In the Wistar rat, the eosinophilic type is very common.

Table 7: Incidence and severity of microscopic changes in the liver-all animals, carcinogenicity phase in rats

Sex	Males				Females			
penflufen Dose level (ppm)	0	100	2000	7000	0	100	2000	7000
Number of examined animals	60	60	60	60	60	60	60	60
Hepatocellular carcinoma								
Total	1	1	0	0	0	0	1	0
%					0%	0%	1.7%	0%
HCD	2				0			
Max%	3.3%				0%			
Hepatocellular adenoma								
Total	1	1	0	2	0	2	5*	4
%					0%	3.4%	8.3%	6.7%
HCD	3				3			
Max %	5%				5%			
Eosinophilic focus(i) of hepatocellular alteration								
Minimal	22	26	26	20	25	24	23	23
Slight	1	4	6	9	2	14	22	15
Moderate	0	0	0	1	0	0	1	1
Total	23	30	32	30	27	38	46**	39*
%					45%	63.3%	76.7%	65%
HCD	47				33(/55)			
Max %	78.3%				60%			
Hepatocellular hypertrophy: centrilobular to panlobular								
Minimal	0	5	20	39	0	0	21	26
Slight	0	0	1	11	0	0	1	20
Moderate	0	0	0	0	0	0	0	1
Total	0	5*	21**	50**	0	0	22**	47**

Altogether these findings show that there was no evidence of treatment-related increased incidence of neoplastic findings following a 2-year exposure period with penflufen which is thus considered not to be carcinogenic in the Wistar rat.

Proposed mechanism of action

To elucidate the mechanism of action three studies were performed.

In the first study performed on cultured female rat hepatocytes (██████████, 2011a), the enzyme and DNA-induction were investigated. Stimulation of cytochrome P450 2B and 3A activities (CYP2B and CYP3A) and cell proliferation (measured as the change in replicative DNA synthesis [S-phase of the cell cycle]) was determined. Phenobarbital sodium salt (PB) and epidermal growth factor (EGF) were included as positive controls for induction of CYP2B/3A activities and cell proliferation, respectively.

The activity of CYP2B in cultured hepatocytes was determined by the formation of resorufin from pentoxyresorufin (PROD activity). The activity of CYP3A in cultured hepatocytes was determined by the formation of resorufin from benzyloxyresorufin (BROD activity). The activity of CYP3A1 in cultured hepatocytes was determined by the formation of 7-hydroxyquinoline from benzyloxyquinoline (BQ activity). The number of cells undergoing replicative DNA synthesis (S-phase) in any given hepatocyte population can be determined by the incorporation of BrdU followed by immunostaining. The number of hepatocytes undergoing DNA synthesis was recorded as the labelling index [(no. of labelled hepatocytes /total no. of hepatocytes) x 100].

The data showed that culturing primary female rat hepatocytes with penflufen produced an increase in replicative DNA synthesis of up to a maximum of approximately 1.7–fold (Table 8). Similar increases were observed following exposure of hepatocytes to phenobarbital. Culturing primary rat hepatocytes with penflufen resulted in an increase in pentoxyresorufin-*O*-depentylation (PROD) activity of up to a maximum of 5.7 fold and a small increase in benzyloxyresorufin-*O*-debenzylation (BROD) activity, up to a maximum of 1.8- fold. This indicates that penflufen is an inducer of CYP2B and CYP3A, respectively. Culturing primary rat hepatocytes with penflufen resulted in an increase in benzyloxyquinoline-*O*-debenzylation (BQ) activity, up to a maximum of 2.4 fold. This indicates that penflufen is an inducer of CYP3A. Phenobarbital administration showed similar magnitudes of PROD, BROD and BQ induction.

Table 8: Replicative DNA synthesis, pentoxyresorufin-O-depentylation (PROD), benzyloxyresorufin-O-debenzylation (BROD) and benzyloxyquinoline-O-debenzylation (BQ) activities in cultured female rat hepatocytes

Treatment	Replicate DNA Synthesis Labelling Index (%)	PROD (pmol resorufin formed/min/mg protein)	BROD (pmol resorufin formed/min/mg protein)	BQ (nmol 7-Hydroxyquinoline formed/min/mg protein)
Vehicle Control	7.22 ± 1.14 # (100.0 ± 15.8) ^a	0.036 ± 0.007 # (100.00 ± 19.55) ^b	0.524 ± 0.062 # (100.00 ± 11.90) ^b	0.037 ± 0.002 # (100.00 ± 6.61) ^b
PB 10 µM	11.62 ± 1.26*** (161.0 ± 17.5)	0.093 ± 0.022* (255.54 ± 60.52)	0.768 ± 0.025** (146.54 ± 4.79)	0.057 ± 0.008* (155.45 ± 20.51)
PB 100 µM	13.50 ± 1.46*** (187.1 ± 20.2)	0.367 ± 0.070** (1014.38 ± 192.13)	2.990 ± 0.927** (570.20 ± 176.67)	0.114 ± 0.034* (308.45 ± 93.30)
PB 1000 µM	12.39 ± 1.44*** (171.6 ± 19.9)	0.334 ± 0.042*** (921.12 ± 116.10)	2.553 ± 0.196*** (486.78 ± 37.38)	0.309 ± 0.023*** (839.99 ± 61.45)
Penflufen 0.1 µM	12.34 ± 0.99*** (171.0 ± 13.8)	0.206 ± 0.055** (569.78 ± 152.02)	0.585 ± 0.042 (111.46 ± 7.98)	0.056 ± 0.006** (150.87 ± 17.20)
Penflufen 1 µM	11.38 ± 1.19*** (157.7 ± 16.5)	0.124 ± 0.038* (343.24 ± 105.92)	0.569 ± 0.034 (108.56 ± 6.56)	0.048 ± 0.001** (129.79 ± 1.40)
Penflufen 3 µM	11.64 ± 1.43*** (161.2 ± 19.9)	0.067 ± 0.016* (185.73 ± 44.38)	0.567 ± 0.162 (108.13 ± 30.80)	0.044 ± 0.002* (120.05 ± 6.22)
Penflufen 10 µM	9.41 ± 0.96* (130.4 ± 13.3)	0.093 ± 0.009** (255.42 ± 24.92)	0.745 ± 0.143 (142.13 ± 27.25)	0.058 ± 0.003*** (156.36 ± 7.82)
Penflufen 30 µM	7.44 ± 0.91 (103.1 ± 12.6)	0.068 ± 0.009** (187.79 ± 25.06)	0.967 ± 0.077** (184.30 ± 14.66)	0.085 ± 0.011** (231.52 ± 28.63)
Penflufen 100 µM	6.55 ± 1.14 (90.7 ± 15.8)	0.089 ± 0.054 (244.35 ± 149.57)	0.674 ± 0.041* (128.44 ± 7.82)	0.089 ± 0.004*** (242.92 ± 12.21)
EGF 25 ng/mL	40.00 ± 4.20*** (554.3 ± 58.2)			

^a Values are Mean ± SD. #Values in parenthesis are mean % control ± SD; ^a (n = 5 per group); ^b (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.

In conclusion, these data showed that penflufen seems to exhibit the general properties of a “phenobarbital-like” inducer acting *via* CAR (and possibly PXR).

In the second study (██████████, 2011b), the potential of penflufen to induce cytochrome P450 2B and 3A activities (CYP2B and CYP3A) and cell proliferation (measured as the change in replicative DNA synthesis [S-phase of the cell cycle]) in isolated female human hepatocyte cultures was investigated.

Phenobarbital sodium salt (PB) and epidermal growth factor (EGF) were included as positive controls

for induction of CYP2B/3A activities and cell proliferation, respectively.

The activity of CYP2B in cultured hepatocytes was determined by the formation of resorufin from pentoxyresorufin (PROD activity). The activity of CYP3A in cultured hepatocytes was determined by the formation of resorufin from benzyloxyresorufin (BROD activity). The activity of CYP3A1 in cultured hepatocytes was determined by the formation of 7-hydroxyquinoline from benzyloxyquinoline (BQ activity). The number of cells undergoing replicative DNA synthesis (S-phase) in any given hepatocyte population can be determined by the incorporation of BrdU followed by immunostaining. The number of hepatocytes undergoing DNA synthesis was recorded as the labelling index [(no. of labelled hepatocytes /total no. of hepatocytes) x 100].

The data showed that exposure of human hepatocytes to 25ng/mL EGF resulted in a 9-fold increase in replicative DNA synthesis, indicating that the hepatocytes could proliferate following exposure to proliferative stimuli, and therefore demonstrating their suitability for use in investigations involving assessing induction of proliferation (Table 9).

Culturing primary human hepatocytes for 96 hours to phenobarbital resulted in an increase in PROD activity to a maximum of 2.6-fold. No significant increases in PROD were observed following treatment with penflufen. Culturing primary human hepatocytes for 96 hours with phenobarbital or penflufen resulted in a dose-dependent increase in BROD activity up to a maximum of 5-fold or 1.45-fold, respectively. In addition, culturing primary human hepatocytes, for 96 hours, with phenobarbital or penflufen resulted in a dose-dependent increase in BQ activity of up to 3.3-fold or 2-fold, respectively (Table 9).

Table 9: Replicative DNA synthesis, pentoxyresorufin-O-depentylation (PROD), benzyloxyresorufin-O-debenzylation (BROD) and benzyloxyquinoline-O-debenzylation (BQ) activities in cultured female human hepatocytes

Treatment	Replicate DNA Synthesis Labelling Index (%)	PROD (pmol resorufin formed/min/mg protein)	BROD (pmol resorufin formed/min/mg protein)	BQ (nmol 7-Hydroxyquinoline formed/min/mg protein)
Vehicle Control	0.608 ± 0.169 # (100.0 ± 27.8) ^a	0.054 ± 0.015 # (100.00 ± 26.8) ^b	0.271 ± 0.037 # (100.00 ± 13.6) ^b	0.171 ± 0.022 # (100.00 ± 12.75) ^b
PB 10 µM	0.483 ± 0.155 (79.5 ± 25.5)	0.064 ± 0.030* (117.8 ± 54.2)	0.259 ± 0.003 (95.6 ± 1.1)	0.223 ± 0.067 (130.21 ± 38.91)
PB 100 µM	0.449 ± 0.124 (73.9 ± 20.4)	0.056 ± 0.031 (102.6 ± 57.0)	0.415 ± 0.051* (153.2 ± 18.9)	0.333 ± 0.028** (194.26 ± 16.59)
PB 1000 µM	0.547 ± 0.209 (90.1 ± 34.4)	0.142 ± 0.051 (261.2 ± 94.2)	1.364 ± 0.041*** (503.6 ± 15.3)	0.562 ± 0.087** (328.31 ± 50.55)
Penflufen 0.1 µM	0.532 ± 0.096 (87.5 ± 15.8)	0.040 ± 0.022 (72.5 ± 39.5)	0.293 ± 0.035 (108.0 ± 13.0)	0.225 ± 0.015* (131.21 ± 8.81)
Penflufen 1 µM	0.530 ± 0.117 (87.2 ± 19.2)	0.043 ± 0.040 (78.2 ± 72.6)	0.286 ± 0.062 (105.6 ± 23.0)	0.191 ± 0.057 (111.72 ± 33.16)
Penflufen 3 µM	0.536 ± 0.121 (88.2 ± 19.9)	0.084 ± 0.015 (153.8 ± 27.8)	0.365 ± 0.107 (134.6 ± 39.6)	0.272 ± 0.027** (158.74 ± 15.68)
Penflufen 10 µM	0.623 ± 0.126 (102.5 ± 20.7)	0.031 ± 0.030 (56.5 ± 55.2)	0.393 ± 0.025** (145.0 ± 9.1)	0.309 ± 0.033** (180.52 ± 19.09)
Penflufen 30 µM	0.615 ± 0.155 (101.3 ± 25.5)	0.021 ± 0.002** (38.49 ± 3.11)	0.246 ± 0.042 (91.0 ± 15.4)	0.337 ± 0.061* (196.78.52 ± 35.87)
Penflufen 100 µM	0.604 ± 0.073 (99.4 ± 12.1)	0.031 ± 0.015 (56.34 ± 27.08)	0.204 ± 0.065 (75.36 ± 23.98)	0.328 ± 0.014*** (191.33 ± 8.05)
EGF 25 ng/mL	5.476 ± 0.232*** (901.3 ± 38.2)			

^a Values are Mean ± SD. #Values in parenthesis are mean % control ± SD; ^a (n = 5 per group); ^b (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.

These data showed that penflufen and phenobarbital appeared to exhibit similar properties in human hepatocytes, inducing cytochromes P450 *via* the Pregnane X Receptor (PXR) and possibly the Constitutive Androstane Receptor (CAR). This was demonstrated by little or no PROD (CYP2B) induction, slightly better BROD (CYP2B/CYP3A) induction and even stronger induction of BQ (CYP3A) by both compounds. No DNA synthesis has been observed following treatment with either

penflufen or the reference compound phenobarbital.

Penflufen is a “phenobarbital-like” inducer in human hepatocytes. As expected, these human receptors did not mediate compound-stimulated DNA synthesis in human hepatocytes as previously observed in rat hepatocytes.

In the third mechanistic study (BCS 2013, M-444600-01-1), penflufen was administered continuously via the diet to groups of Wistar female rats for at least 7 days at concentrations of 7000 ppm (equating approximately to 595 mg/kg bw/day). A similarly constituted group received untreated diet and acted as a control. An additional group received phenobarbital by oral gavage once per day at 80 mg/kg/day and acted as positive control for enzyme induction. Gene transcript analyses were conducted by Quantitative Polymerase Chain Reaction for the following enzymes and proteins: CYP 1A1, CYP 2B1, CYP 3A3, CYP 4A1, UDPGTR2, UGT 1A6, SULT 2A2, EPHX 1, GSTM 4 and POR. Liver cell proliferation as well as total cytochrome P-450 content, cytochrome P-450 isoenzyme and UDPGT activities (bilirubin and 4-nitrophenol) were also investigated.

Following treatment with penflufen, the assessment of cell proliferation in the liver revealed a higher BrdU labeling index in the centrilobular and the periportal area, when compared to the controls. The overall BrdU labeling index (centrilobular + periportal) was higher than the controls. Following phenobarbital administration, the assessment of cell proliferation in the liver revealed a higher BrdU labeling index in the centrilobular area, when compared to the controls. The overall BrdU labeling index (centrilobular + periportal) was higher than the controls (Table 10).

Table 10: Liver cell proliferation assessment following penflufen or phenobarbital administration in the female rats

		Centrilobular	Periportal	Total
Control	N	5	5	5
	Mean	54.49	41.85	48.17
	STD	38.98	12.41	25.54
Phenobarbital	N	5	5	5
	Mean	80.90	41.46	61.18
	STD	22.77	30.01	25.50
Penflufen	N	5	5	5
	Mean	87.06	67.14	77.10
	STD	47.79	37.57	40.98

Regarding the hepatotoxicity testing, total cytochrome P-450 content was increased following penflufen and phenobarbital administration, when compared to the controls. As for phenobarbital, phase I enzymatic activities (BROD and PROD) and phase II enzymatic activities (UDPGT 4-nitrophenol and UDPGT bilirubin) were increased after penflufen treatment and when compared to the control group (Table 11).

Table 11: Total cytochrome P450 and enzymatic activities in the liver following administration of penflufen or phenobarbital in female rats

	Total cytochrome P-450 content and enzymatic activities Mean ± standard deviation (% compared to controls)		
	Control	Phenobarbital 80 mg/kg/day	Penflufen 7000 ppm 595 mg/kg/day
P-450 (nmol/mg Prot.)	1.11 ± 0.11	1.53* ± 0.28 (38%)	1.79** ± 0.15 (61%)
EROD (pmol/min/ mg Prot.)	36.29 ± 10.92	38.57 ± 2.46	41.71 ± 4.92
PROD (pmol/min/ mg Prot.)	3.16 ± 0.61	28.75* ± 19.85 (810%)	11.60** ± 3.76 (267%)
BROD (pmol/min/ mg Prot.)	3.01 ± 1.10	117.07 ± 94.75 (3789%)	50.214* ± 23.52 (1568%)
LAH (nmol/min/ mg Prot.)	4.85	4.62	4.75
UDPGT-4Nitrophenol (pmol/min/ mg Prot.)	6.31 ± 0.34	12.87** ± 3.07 (104%)	17.16** ± 3.55 (172%)
UDPGT-Bilirubin (pmol/min/ mg Prot.)	0.57 ± 0.29	1.06** ± 0.11 (86%)	2.15** ± 0.25 (277%)

The means and standard deviation are calculated with 4 pools of 5 animals in each group

** : statistically different from the control group (p≤0.01)

* : statistically different from the control group (p≤0.05)

Following treatment with penflufen at 7000 ppm, the most markedly up-regulated phase I enzyme gene transcripts in the liver were Cyp3a3 and Cyp2b1 when compared to the controls. These increases were also observed after phenobarbital treatment.

An increase in Cyp1a1 gene transcripts was observed after penflufen treatment unlike after phenobarbital treatment, nevertheless this increase was not translated in an increase in EROD enzymatic activities.

Cyp4a1 gene transcripts were slightly down-regulated with both penflufen and phenobarbital.

Regarding the phase II enzymes, Udpgr2, Ugt1a6, Gstm4 and Ephx1 were up-regulated following penflufen or phenobarbital administration, when compared to the controls (Table 12). Por gene transcripts were slightly down-regulated following both penflufen and phenobarbital administration.

Table 12: Mean relative quantity of gene transcripts in the liver following penflufen or phenobarbital administration in female rats

Gene transcripts	Mean Relative Quantity ± standard deviation of gene transcripts (% change compared to control mean values)		
	Control	Phenobarbital 80 mg/kg/day	Penflufen 7000 ppm
Cyp1a1	1.22 ± 0.788	1.01 ± 0.617	6.24** ± 4.970 (+411%)
Cyp2b1	1.12 ± 0.126	31.89** ± 22.489 (+2747%)	7.32** ± 5.289 (+554%)
Cyp3a3	0.39 ± 0.365	3.46** ± 2.120 (+787%)	4.48** ± 2.227 (+1049%)
Cyp4a1	1.23 ± 0.433	0.71 ± 0.264	1.00 ± 0.231
Ugt1a6	1.05 ± 0.305	3.36** ± 1.646 (+220%)	2.88* ± 1.838 (+174%)
Udpgr2	0.98 ± 0.224	2.62** ± 1.381 (+167%)	2.75** ± 0.805 (+181%)
Sult2a2	0.96 ± 0.284	0.52* ± 0.163 (-46%)	1.42 ± 0.522 (+48%)
Ephx1	0.95 ± 0.322	3.02** ± 1.086 (+218%)	1.97* ± 0.681 (+107%)
Gstm4	1.35 ± 0.654	9.85** ± 8.257 (+630%)	2.82 ± 2.433
Por	1.25 ± 0.309	0.62** ± 0.110 (-50%)	0.86* ± 0.144 (-31%)

** : statistically different from the control group (p≤0.01)

* : statistically different from the control group (p≤0.05)

Discussion

The dietary administration of penflufen produced higher incidence of hepatocellular adenoma (HCA) in female rats following a 2-year treatment period. Given that these HCA were not dose-related in rats and taken into account the lack of genotoxicity potential of penflufen, the higher incidence of HCA could be considered to be subsequent to a threshold mechanism with a phenobarbital-like mechanism of action (hepatocellular hypertrophy and transient cell proliferation) which is a well known mechanism of action specific to the rodent and of no relevance to humans (Anderson *et al.*, 1992, Grasso, P. *et al.* 1991, ██████████ *et al.* 2013).

In *in vitro* explanatory toxicity studies, penflufen was shown to be an inducer of total cytochrome P450 and BROD, PROD and BQ associated activities in female rat and human hepatocytes. Penflufen and phenobarbital induced DNA synthesis only in rat hepatocytes supporting that a mitogenic response would not occur in humans. These findings were similar to those observed with phenobarbital showing that penflufen is a phenobarbital-like compound.

In the 7-day explanatory toxicity study performed in female rats, penflufen at 7000 ppm induced BROD and PROD enzymatic activities as well as an increase in Cyp2b, Cyp3a, Udpgr2 and Ugt1a6 gene transcripts as phenobarbital did. These data indicate that CAR/PXR receptors were indeed activated by penflufen which possibly lead to the observed liver cell proliferation following continuous dietary administration for at least 7 days in female Wistar rats.

Many nongenotoxic xenobiotics that stimulate hepatomegaly in the short term have been found to produce liver tumours following long-term administration to rodents (Allen *et al.*, 2004; Grasso *et al.*, 1991; Schulte-Hermann *et al.*, 1980). Tumours induced by nongenotoxic chemicals are generally only produced by long-term administration of doses frequently exceeding human exposures by orders of magnitude. Additionally, the mitogenic response to the prototypical enzyme inducer, phenobarbital (PB) (and similar inducers) appears to be absent in man (Lake, 2009). Thus, the significance of rodent liver tumours to human risk assessment is therefore unclear. However, despite PB and related compounds exhibiting nongenotoxic hepatocarcinogenicity and having the properties of liver tumour promoters in rats and mice, they do not appear to produce liver tumours in humans. A number of epidemiological studies have demonstrated that in human subjects receiving PB for many years, at

doses producing plasma concentrations similar to those that are carcinogenic in rodents, there is no evidence of increased liver tumour risk (IARC, 2001; Olsen *et al.*, 1989; Whysner *et al.*, 1996).

Penflufen is a “phenobarbital-like” enzyme inducer in both human and rat hepatocytes. It induces CYP2B and CYP3A, with CYP2B preferentially induced in rat hepatocytes and CYP3A being preferentially induced in human hepatocytes. This pattern is frequently observed and implies a CAR/PXR mechanism of action involvement.

As described in the *in vitro* explanatory toxicity studies, while both hypertrophic (enzyme induction) and hyperplastic (S-phase) effects were seen in rat hepatocytes, only the enzyme induction was observed in human hepatocytes. Therefore and since S-phase stimulation was not observed in human hepatocytes, hyperplasia is not expected to occur in human.

Therefore, if the CAR/PXR receptor-mediated stimulation of cell proliferation is pivotal to non-genotoxic hepatocarcinogenesis, then penflufen is unlikely to pose a hepatocarcinogenic hazard to humans.

7.Overall conclusion

These data show that following:

- A long term dietary administration of penflufen to male and female C57/Bl6 mice, a slightly higher incidence of adenoma and carcinoma in the liver was found in all doses in males, when compared to controls. However, these neoplastic findings were found without any dose effect relationship over a quite large range of dose levels. They were not associated with an increased incidence of pre-neoplastic changes. Overall, the incidences of those tumours were only marginally outside the historical control range for this strain of mice and this laboratory. Therefore, these liver tumours were considered not to be treatment-related. In females there was no increased incidence of hepatocellular tumour.
- A long term dietary administration of penflufen to male and female Wistar rats, numerically higher incidences of neoplastic lesions were observed in the liver. The hepatocellular adenoma observed in female rats following a 2-year treatment period with penflufen was considered to be subsequent to a phenobarbital-like mechanism of action which is a well known mechanism of action specific to the rodent and of no relevance to humans.
- A long term dietary administration of penflufen to male and female Wistar rats, numerical higher incidences of neoplastic lesions were observed in ovaries, hematopoietic system and brain in some treated dose groups, generally the high dose group. Given that these incidences were similar to internal and/or external historical control data, that these neoplastic lesions were generally not dose-related and that penflufen is devoid of any genotoxic potential, it was concluded that the neoplastic lesions found in the ovaries, hematopoietic system and brain were not related to treatment with penflufen.

Altogether, these findings clearly show that penflufen is not considered to present a carcinogenic risk to humans.

Therefore Bayer proposes to not classify penflufen with H351 (cat.2 carcinogenic).

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Abbreviations

BCS = Bayer CropScience

BROD = benzyloxyresorufin-O-debenzylation

BQ = benzyloxyquinoline

CYP: cytochrom P-450

EROD = ethoxy-resorufin-O-deethylation

HAC = hepatocellular adenoma

ppm = Parts per million

PROD = pentoxy-resorufin-O-depentylation

P-450 = cytochrom P-450

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