Benfluralin

Mode of action and human relevance of rodent liver and thyroid tumors

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[redacted]

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1. Abstract

This paper addresses the human relevance of liver and thyroid tumors seen in long-term studies with benfluralin in rats and mice and presents a proposed mode of action for liver and thyroid tumors according to the framework developed by the International Programme on Chemical Safety (IPCS; Boobis *et al*, 2006).

Benfluralin is a herbicide of the dinitroaniline class which is used to control grasses and other weeds.

Increased incidences of

- hepatocellular adenoma and carcinoma in the liver in males and of
- thyroid follicular adenoma and carcinoma in both sexes

were observed in a combined chronic toxicity and carcinogenicity study in F344 rats ([redacted], 1996), and

- increased incidence of hepatocellular adenomas and carcinomas at the highest dose in both sexes of mice in a carcinogenicity study [redacted] 1988).

Genotoxicity as a potential mode of action (MOA) for both tumor types could be confidently excluded based on a complete data package *in vitro* and *in vivo* addressing mutagenicity and clastogenicity. PPAR-alpha mediated liver enzyme induction could be excluded since the peroxisome proliferator-activated receptor alpha (PPARα)-related transcript, Cyp4a1 was not altered in an available mode of action study ([redacted], 2010). The herbicidal mechanism of action of benfluralin (inhibition of root and shoot development) can also be excluded as a potential tumor MOA in mammals.

Since in rats and mice the liver was a main target, increased metabolic enzyme induction as underlying mechanism for the liver and thyroid tumors is highly likely. An available dietary 14-day mechanistic study in rats ([redacted], 2010) indicated increased liver and thyroid weights, hepatocellular and thyroid follicular hypertrophy, hepatic phase I enzyme induction, reduced T4 levels, and phase II (UDPGT) -induction and thus supported a rodent-specific liver enzyme-mediated MOA for liver and thyroid tumor induction. T4 decrease and subsequent TSH increase at doses at which liver effects occurred was also noted in a male and female pubertal assay ([redacted] 2012). The rodent specificity was supported by the fact that no thyroid effects were seen in dogs and by the negative outcome of an Amphibian metamorphosis assay (AMA; [redacted] 2011).

In a follow-up program of mechanistic *in vitro* and *in vivo* studies some elements deemed by regulatory authorities to be necessary to investigate were addressed.

Liver tumor MOA

In vitro studies in cultured hepatocytes from wildtype and CAR/PXR-double knockout rats treated with benfluralin (Chatham 2019) support this MOA. A proliferation of hepatocytes was only observed in wildtype rats, but not in CAR/PXR-knockout rats, thus, demonstrating that a CAR/PXR-mediated mechanism is responsible for the liver tumor induction of benfluralin. Importantly, *in vitro* studies in cultured hepatocytes from human donors (Chatham 2019) did not show proliferation. This MOA is proposed for the liver tumors in the rodent studies with benfluralin; this MOA is known to be of no relevance to humans (Elcombe et al., 2014).

Thyroid tumor MOA

Findings were limited to rodents; no comparable effects were observed in dogs, where a full database with 3 studies from 90 day- to 1 year-treatment exists. An investigation on a direct effect on the thyroid by inhibition of the Thyroid Peroxidase (TPO) enzyme in rat thyroid microsomes did not show any inhibition of TPO activity by benfluralin (Richert 2019). This MOA can be confidently excluded. According to a publication about testing of many compounds, including benfluralin on sodium iodide symporter (NIS) inhibition, benfluralin was negative, so that also based on this result no evidence of a direct thyroid effect was detected (Wang et al, 2018).

A new 13-week study in male and female F344 rats with oral administration of benfluralin via diet ([redacted] 2019) led to a decrease of T4 (already observed in the existing 14-day study), but also demonstrated an increase of TSH (and T3) as postulated.

Altogether these results observed in the rats exclude a direct effect on the thyroid and support a liver enzyme-mediated mechanism via increased T4 metabolism due to induction of UDPGT. The subsequently lower T4 levels trigger a corresponding increase of TSH levels which lead to follicular cell hypertrophy/hyperplasia and finally in rodents after lifetime exposure to thyroid tumors. This MOA is proposed for the thyroid tumors in the rat study with benfluralin; this MOA is known to be of no relevance to humans (Dellarco et al., 2006).

2. Introduction

In a combined chronic toxicity and carcinogenicity study of benfluralin in F344 rats with dietary concentrations of 0, 10, 100, 2500 and 5000 ppm for 24 months increased incidences of hepatocellular adenoma and carcinoma in the liver in males and of thyroid follicular adenoma and carcinoma in both sexes at 2500 and 5000 ppm were observed at the end of the 104-week study. An overview of the liver tumors is given in tables 1 for rats and table 2 for mice, and for the thyroid tumor incidences in table 2.

Table 1: Overview of the incidence of liver tumors in the long-term rat study ([redacted], 1996)

Dose (ppm)	0	10	100	2500	5000
Liver					
Males					
Number of animals examined week 52	10	10	10	10	10
Number of animals of carcinogenicity phase (week 104)	48	50	48	50	50
Hepatocellular adenoma week 52	0	0	0	1	0
Hepatocellular adenoma week 104	1	2	1	3	9
Hepatocellular carcinoma week 104	1	0	0	2	2
Combined hepatocellular adenoma and carcinoma	2	2	1	6	11*
Females					
Number of animals examined week 52	10	10	10	10	10
Number of animals examined week 104	50	50	50	50	50
Hepatocellular adenoma week 52	0	0	0	0	0
Hepatocellular adenoma week 104	2	0	1	1	2
Hepatocellular carcinoma week 104	0	0	0	0	0
Combined hepatocellular adenoma and carcinoma	2	0	1	1	2

Incidences as number of animals with tumor
Fisher-Irwin exact test *p<0.05

Table 2: Overview of thyroid tumors in the long-term rat study ([redacted], 1996)

Dose (ppm)	0	10	100	2500	5000
Thyroid					
Males					
Number of animals examined week 52	10	10	10	10	10
Number of animals examined week 104	48	50	48	50	50
Follicular cell adenoma week 52	0	0	0	0	1
Follicular cell adenoma week 104	1	1	1	3	5
Follicular cell carcinoma week 52	0	0	0	1	0
Follicular cell carcinoma week 104	0	0	0	4	3
Combined follicular cell adenoma and carcinoma	1	1	1	8*	9*
Females					
Number of animals examined week 52	10	10	10	10	10
Number of animals examined week 104	50	50	50	50	50
Follicular cell adenoma week 52	0	0	0	1	0
Follicular cell adenoma week 104	0	0	0	3	2
Follicular cell carcinoma week 52	0	0	0	0	0
Follicular cell carcinoma week 104	0	0	1	2	2

Combined follicular cell adenoma and carcinoma	0	0	1	6	4

Incidences as number of animals with tumor
Fisher-Irwin exact test *p<0.05

In a chronic toxicity and carcinogenicity study in B6C3F1 mice with dietary concentrations of 0, 50, 300 and 1500 ppm for 24 months increased incidences of hepatocellular adenoma and carcinoma in the liver were observed in males and females. A summary is given in the following table.

Table 3: Overview of histopathological liver findings in the long-term study in mice [redacted], 1988)

Dose (ppm)	0	50	300	1500
Number of animals examined week 104	60	60	60	60
Males				
Focal hepatocellular hyperplasia	20	10	11	14
Multifocal hepatocellular hyperplasia	1	2	5	8
Hepatocellular adenoma	2	1	3	4
Hepatocellular carcinoma	7	7	5	8
Combined hepatocellular adenoma and carcinoma	9	8	6	12
Females				
Focal hepatocellular hyperplasia	6	5	4	15
Multifocal hepatocellular hyperplasia	1	0	1	8
Hepatocellular adenoma	1	1	1	3
Hepatocellular carcinoma	0	2	3	3
Combined hepatocellular adenoma and carcinoma	1	3	4	6

Incidences as number of animals with tumor

An extensive testing program was performed with the objective to investigate the MOA of these tumors. The MOA studies and their results are summarized in this paper.

3. MOA study results

3.1 Liver tumor MOA

3.1.1 *In vivo* studies

3.1.1.1 14-day study in rats ([redacted], 2010)

Results of a 14-day study in the same rat strain as used in the long-term rat study, i.e. F344 rats with doses of 0 and 5000 ppm which was the carcinogenic dose in the long-term study, are available. The results indicated increased liver and thyroid weights, hepatocellular and thyroid follicular hypertrophy, hepatic phase I enzyme induction, predominantly of CYP2B with an increase of 300- to 500-fold based on mRNA and of 5- to 7-fold based on protein activity level. Also, an induction of CYP3A was seen, with mRNA level increases by a factor of 10. Furthermore, reduced T4 levels were seen.
This outcome clearly supported the proposed liver enzyme-mediated MOA for liver and thyroid tumor induction.

3.1.1.2 *In Vivo* Hepatocellular Proliferation Studies

Studies were planned to address *in vivo* cell proliferation. Due to the timing of the classification process in Europe, they were not available at the time of public consultation.

3.1.2. *In vitro* studies

3.1.2.1 *In vitro* studies in cultured hepatocytes from Sprague-Dawley wildtype, knockout and F344 rats, B6C3F1 mice and from human donors

The objective of these studies was to investigate the potential of benfluralin to stimulate cell proliferation in the hepatocytes of laboratory rodent species, i.e. wildtype rats and mice in comparison with human hepatocytes to assess the relevance of the liver effects in the laboratory species to humans. Furthermore, proliferation was also measured in CARKO/PXRKO (Constitutive androstane receptor-knockout/ pregnane X receptor-knockout) rat hepatocytes to evaluate the relevance of CAR and PXR for the liver tumor MOA since in the CARKO/PXRKO cells no proliferation would be expected.
Therefore, the proliferation potential of benfluralin (measured as the change in replicative DNA synthesis, referred to as S-phase) was determined in cultures of primary hepatocytes isolated from

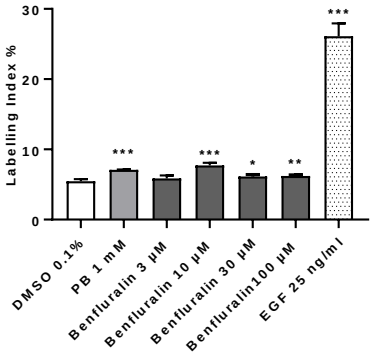
- B6C3F1 mice (strain in which the carcinogenicity study was conducted),
- male F344 rats (strain in which the carcinogenicity study was conducted),
- from Sprague Dawley rats (strain from whose genetic background CAR/PXR knockout rats are available; both wild type and KO were used) and
- from human donors.

Before the proliferation studies, cytotoxicity was determined by measuring adenosine 5´-triphosphate (ATP) levels in all wells as a function of ATP levels in control samples. Concentrations up to the limit of cytotoxicity were used in these studies. EGF (epidermal growth factor) served as positive control. Details are given in Appendix 1.

Induction of DNA synthesis in cultured male F344 rat hepatocytes (Chatham, 2019a)

Treatment with benfluralin stimulated cell proliferation in cultures of primary hepatocytes isolated from F344 rats at 10, 30 and 100 µM with a maximum induction of 1.4-fold (10 µM) as shown in the following figure.

Figure 1: Effect of benfluralin, PB or EGF on S-Phase in primary hepatocytes prepared from male F344 rats

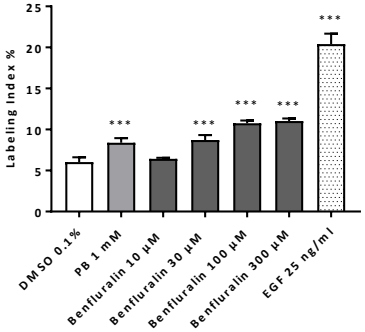


Labelling index (%) was used to determine S-Phase for rat hepatocytes treated with benfluralin, PB, EGF or vehicle control (DMSO). Data are presented as mean ± SD (n = 5). Statistical significance between benfluralin and DMSO control was tested using a one way ANOVA followed by a Dunnett's multiple comparison test. A comparison between DMSO control and PB or EGF treated hepatocytes was performed using a Student' t-test. * statistically different from control $p<0.05$; ** $p< 0.01$; *** $p< 0.001$.

Induction of DNA synthesis in cultured male Sprague-Dawley rat hepatocytes (Chatham, 2019b)

Treatment with benfluralin stimulated cell proliferation in cultures of primary hepatocytes isolated from Sprague Dawley rats in a dose dependent manner to a maximum of 1.8-fold at 300 µM, as shown in the following figure.

Figure 2: Effect of benfluralin, PB or EGF on S-Phase in primary hepatocytes prepared from male Sprague Dawley rats



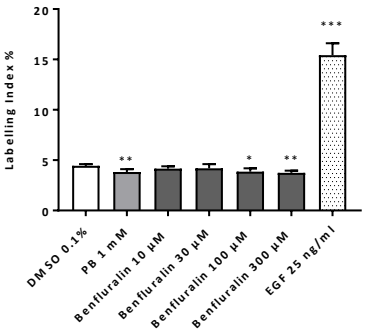
Labelling index (%) was used to determine S-Phase for rat hepatocytes treated with benfluralin, PB, EGF or vehicle control (DMSO). Data are presented as mean ± SD (n = 5). Statistical significance between benfluralin and DMSO control was tested using a one way ANOVA followed by a Dunnett's multiple comparison test. A comparison between DMSO control and PB or EGF treated hepatocytes was performed using a Student' t-test. *** statistically different from control $p < 0.001$.

The reaction of Sprague-Dawley and F344 rat hepatocytes was similar which justifies the use of CARKO/PXRKO hepatocytes from Sprague-Dawley rats.

Induction of DNA synthesis in cultured male CARKO/PXRKO rat hepatocytes (Chatham, 2019c)

The aim of this study was to investigate the potential effect of benfluralin to stimulate cell proliferation in cultures of primary hepatocytes isolated from male Sprague Dawley rats nulled for the constitutive androstane receptor and the pregnane X receptor (CARKO/PXRKO rats). Benfluralin concentrations of up to and including 300 µM did not result in cell proliferation in cultured hepatocytes from CARKO/PXRKO rats which demonstrates clearly the proposed liver tumor MOA via CAR/PXR induction. The results are shown in the following figure.

Figure 3: Effect of benfluralin, PB or EGF on S-Phase in primary hepatocytes prepared from male CARKO/PXRKO rats

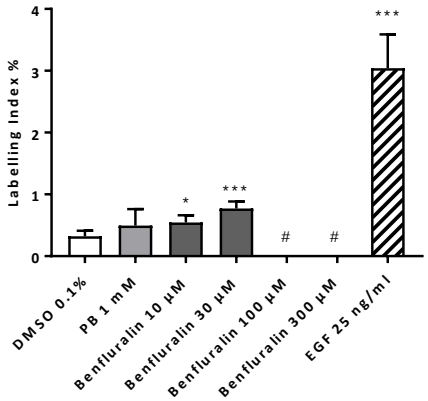


Labelling index (%) was used to determine S-Phase for rat hepatocytes treated with benfluralin, PB, EGF or vehicle control. Data are presented as mean ± SD (n = 5). Statistical significance between benfluralin and DMSO control was tested using a one-way ANOVA followed by a Dunnett's multiple comparison test. A comparison between DMSO control and PB or EGF treated hepatocytes was performed using a Student' t-test. * statistically different from control p<0.05, ** p<0.01, *** p<0.001.

Induction of DNA synthesis in cultured B6C3F1 mouse hepatocytes (Chatham, 2019d,e)

Treatment with benfluralin stimulated cell proliferation in cultures of primary hepatocytes isolated from male B6C3F1 mice in a dose dependent manner to a maximum of approximately 2-fold at 30 µM, as shown in the following figure.

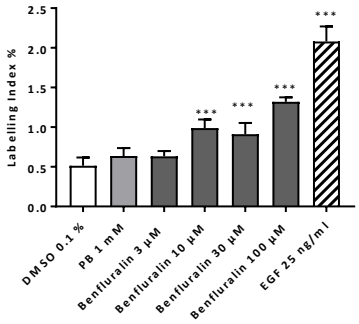
Figure 4: Effect of benfluralin, PB or EGF on S-Phase in primary hepatocytes prepared from male B6C3F1 mice



Labelling index (%) was used to determine S-Phase for mouse hepatocytes treated with benfluralin, PB, EGF or vehicle control. Data are presented as mean ± SD (n = 5). Statistical significance between benfluralin and DMSO control was tested using a one way ANOVA followed by a Dunnett's multiple comparison test. A comparison between DMSO control and PB or EGF treated hepatocytes was performed using a Student' t-test. * statistically different from control p<0.05; *** p<0.001. # indicates that cells were not counted due to toxicity.

Treatment with benfluralin stimulated cell proliferation in cultures of primary hepatocytes isolated from female B6C3F1 mice in a dose dependent manner to a maximum of approximately 2.6-fold at 100 µM., as shown in the following figure.

Figure 5: Effect of benfluralin, PB or EGF on S-Phase in primary hepatocytes prepared from female B6C3F1 mice



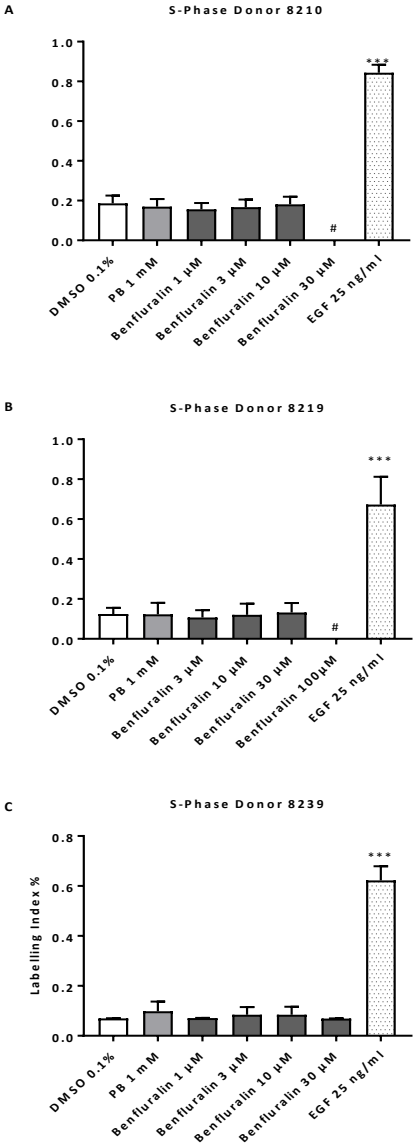
Labelling index (%) was used to determine S-Phase for mouse hepatocytes treated with benfluralin, PB, EGF or vehicle control. Data are presented as mean ± SD (n = 5). Statistical significance between benfluralin and DMSO control was tested using a one-way ANOVA followed by a Dunnett's

multiple comparison test. A comparison between DMSO control and PB or EGF treated hepatocytes was performed using a Student' t-test. *** statistically different from control *** p<0.001.

Induction of DNA synthesis in cultured human hepatocytes (Chatham, 2019f)

In human hepatocytes no proliferation occurred after treatment with benfluralin at concentrations of up to and including 30 µM, based on the data from three donor hepatocyte studies which did not show any proliferation in the hepatocytes of the three donors, 8210, 8219 and 8239 as shown in the following figures.

Figure 6: Effect of benfluralin, PB or EGF on S-Phase in primary hepatocytes prepared from 3 human donors (8210 and 8219 males, 8239 female)



No proliferation was seen in human hepatocytes. The results of a test in another donor (no. 385) which was performed separately from the other three donors were not in line with the toxicity and proliferation data of the three other donors. The study report author concluded that “Although treatment of cultured human hepatocytes with benfluralin at 30- and 300 µM did cause statistically significant increases in cell proliferation, no significant increase was seen at the intervening concentration of 100 µM. On the basis of this lack of dose response, the increase in S-phase labelling by benfluralin was not deemed to be a genuine result.” (Chatham 2019g). Therefore, the use of the data of the first donor 385 is inappropriate for the overall conclusion of the outcome of these tests from a scientific standpoint and can be excluded (details on the study can be found in Appendix 2).
Overall therefore, it is concluded that no proliferation was seen in human hepatocytes which thus demonstrated non-relevance of the rodent liver tumors to humans.

It can be concluded that these *in vitro* studies in cultured hepatocytes from wildtype and CAR/PXR-double knockout rats treated with benfluralin support the postulated liver tumor MOA via CAR and PXR receptor activation and cell proliferation by benfluralin. A proliferation of hepatocytes was only observed in hepatocytes from wildtype rats, but not in CAR/PXR-knockout rats which clearly demonstrates that a CAR/PXR-mediated mechanism is responsible for the liver tumor induction of benfluralin. In addition, cultured hepatocytes from human donors did not show proliferation and thus demonstrated that the rodent-specific MOA of CAR/ PXR-mediated liver tumor induction is not relevant to humans.

3.2 Thyroid MOA

3.2.1 13-Week thyroid study by oral route (dietary formulation) in F344 rats ([redacted], 2019)

The objective of this study was to investigate if 13 weeks of oral administration via dietary admixture of benfluralin was able to decrease T4 and to increase the TSH level as well as liver and thyroid weights as previously described (Study details see Appendix 3).

One group of Fischer 344 rats (10/sex) was administered the test item, benfluralin, via a dietary admixture at a concentration of 10000 ppm for 13 weeks. Another group of 10 males and 10 females received only the untreated diet under the same experimental conditions and acted as a control group.

Thyroid hormone measurements were performed on day 29 and at the end of the treatment period. At necropsy, liver, thyroids with parathyroids, and pituitary gland were weighed and preserved for microscopic examination.

Significantly lower mean T4 concentrations ranging from -57 to -77% *vs.* control for the males and from -52 to -65% *vs.* control for the females after 4 and 13 weeks of treatment were reported concomitantly to an increase in TSH levels (1.9/1.5- and 1.75/2.8-fold induction in week 4 and 13 for males and females respectively *vs.* control values) while T3 hormone levels were slightly increased. The T3/T4 ratio was significantly increased in both sexes (from 3- to 5-fold of control).

The results for males and females were similar, the data for the males are shown in the following figures.

Figure 7: Effect of benfluralin on T4 levels (males)

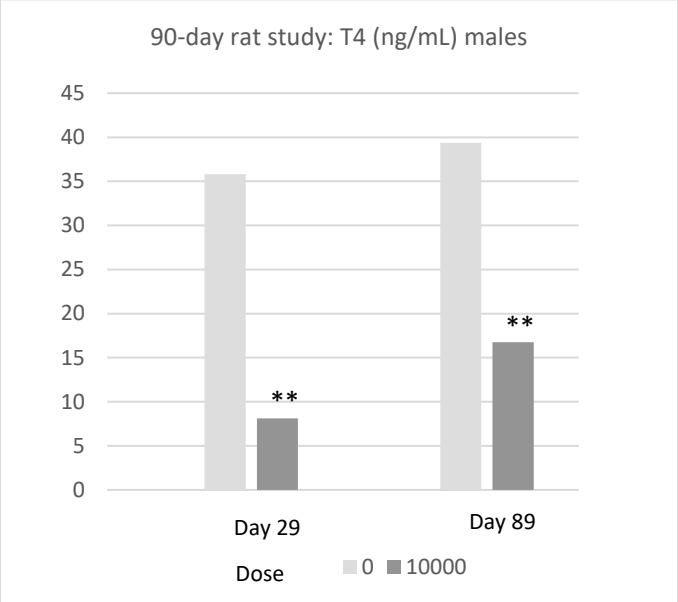


Figure 8: Effect of benfluralin on T3 levels (males)

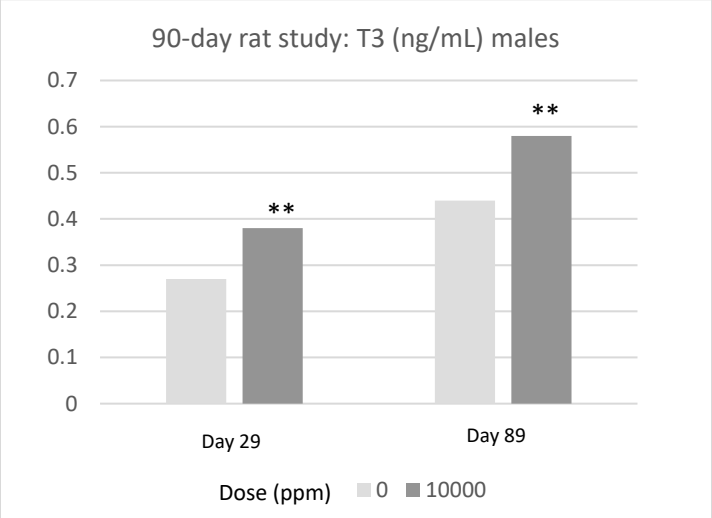


Figure 9: Effect of benfluralin on TSH levels (males)

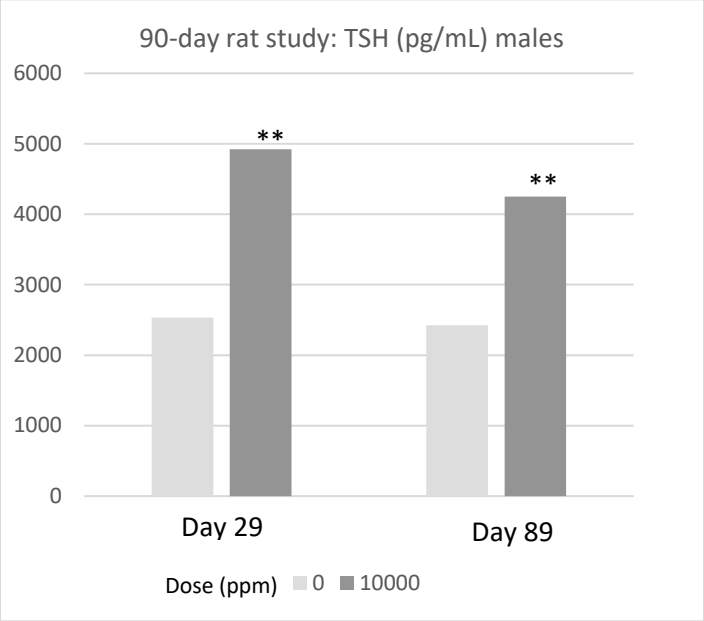
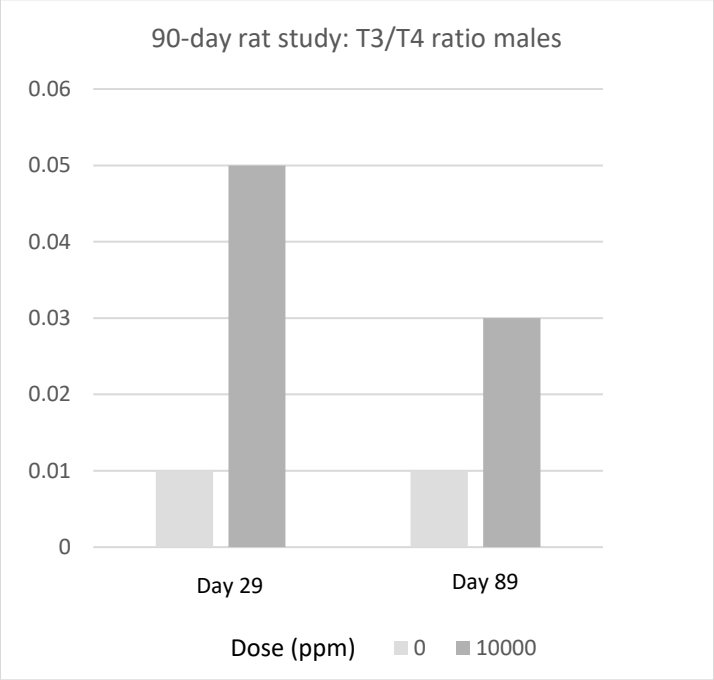


Figure 10: Effect of benfluralin on T3/T4 ratios (males)



Increased liver weights in animals given 10000 ppm Benfluralin were noted in both sexes and correlated with a centrilobular or diffuse hepatocellular hypertrophy in both sexes. Significantly increased thyroid

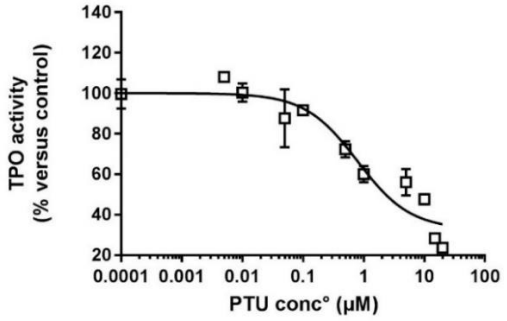
weights were noted in males and a similar trend was noted in females. These changes correlated with the minimal to slight follicular cell hypertrophy/hyperplasia noted in the thyroid gland.

Altogether these results observed in the rats support the proposed liver enzyme-mediated mechanism via increased T4 metabolism and clearance with subsequently lower T4 levels and corresponding increase of TSH levels which after lifetime exposure of rodents leads to follicular cell hypertrophy/hyperplasia and finally to thyroid tumors. This MOA is known to be of no relevance to humans.

3.2.2 Inhibition of Rat microsomal Thyroid Peroxidase by Benfluralin (Richert et al, 2019)

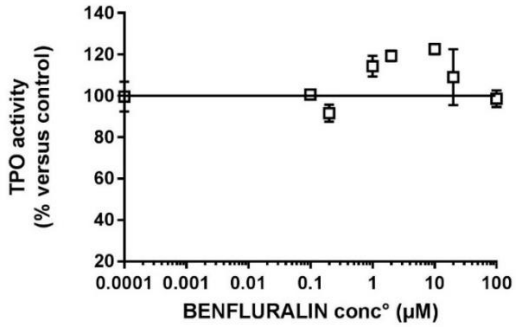
In order to investigate whether a direct effect on the thyroid could have been involved, the potential of benfluralin to inhibit the thyroid peroxidase (TPO) enzyme was investigated in rat thyroid microsomes.
The thyroid peroxidase activity determination was performed using the substrate fluorogenic Amplex UltraRed (AUR) which is converted to fluorescent Amplex UltroRex by TPO in the presence of excess H_2O_2 (see Appendix 4).
The sensitivity of this test system was demonstrated by the reaction of the used positive control, 6-N-Propyl-2-Thiouracil (PTU) as shown in the following figure.

Figure 11: Inhibition curve of Thyroid Peroxidase (TPO) by PTU



In this study, benfluralin did not show any inhibition of TPO activity up to its highest soluble concentration in incubation medium, 100µM, as shown in the following figure.

Figure 12: Inhibition curve of Thyroid Peroxidase (TPO) by Benfluralin



Based on this result, this MOA via inhibition of TPO can be excluded for benfluralin.

3.2.3 Other studies with thyroid effects

Findings were limited to rodents; no comparable effects were observed in dogs, where a full database with 3 studies from 90 day- to 1 year-treatment exists. Other studies which support the proposed thyroid MOA are the male and female pubertal assays [redacted] et al, 2012; [redacted] et al, 2012, both reports in dossier) and the Amphibian metamorphosis assay (AMA) ([redacted] et al, 2011, revised 2012, in dossier). In the male and female pubertal assays, the typical decrease of T4 and the corresponding increase of TSH was observed. Since the thyroid hormone effects were seen at doses at which also liver effects occurred it gives additional support for the proposed liver enzyme-mediated thyroid MOA.
Studies on sodium iodide symporter (NIS) inhibition by many compounds, including benfluralin are described in a publication (Supporting information to Wang et al, 2018). Benfluralin was negative in this test so that based on this result no evidence of a direct thyroid effect was detected.
The AMA was negative, since none of the parameters, wet weights, snout-vent lengths, and hind limb lengths from benfluralin-exposed tadpoles were affected, and also the histopathology of the thyroid did not reveal a treatment-related change. This further supports the rodent specificity of the proposed thyroid MOA.

4. Discussion of MOA data

Benfluralin is a herbicide of the dinitroaniline class which is used to control grasses and other weeds.
In a combined chronic toxicity and carcinogenicity study in F344 rats with dietary concentrations of 0, 10, 100, 2500 and 5000 ppm for 24 months increased incidences of hepatocellular adenoma and carcinoma in the liver were noted in males, and increased thyroid follicular adenoma and carcinoma incidences in both sexes at 2500 and 5000 ppm were observed [redacted], 1996). In a chronic toxicity and carcinogenicity study in mice with dietary concentrations of 0, 50, 300 and 1500 ppm an increased incidence of hepatocellular adenomas and carcinomas was seen at the highest dose of 1500 ppm in both sexes (Koenig, 1988).
Genotoxicity as a mode of action (MOA) could be excluded since the genotoxicity studies did not provide evidence of a genotoxic potential of benfluralin. The mechanism of action of benfluralin which involves inhibition of root and shoot development as a plant-specific mechanism can also be excluded as a potential tumor MOA. PPAR-alpha agonism can be confidently excluded as an alternative mode of action based on the enzyme induction profile determined in rats.
Since in rats at both tumor-inducing concentrations, liver effects, including hepatocellular hypertrophy were seen, increased liver enzyme induction as underlying mechanism for the liver and thyroid tumors was likely. Also, in mice other liver effects at the highest concentration, including hyperplasia were noted.
An available 14-day mechanistic study indicated increased liver and thyroid weights, hepatocellular and thyroid follicular hypertrophy, hepatic phase I enzyme induction, predominantly of CYP2B and reduced T4, and thus supported the proposed MOA for liver and thyroid tumor induction.

Therefore, in a follow-up program of mechanistic in vitro and in vivo studies the MOA of these tumors was further thoroughly investigated.

Liver tumor MOA

In vitro studies in cultured hepatocytes from wildtype and CARKO/PXRKO rats treated with benfluralin supported this MOA since a proliferation of hepatocytes was observed only in wildtype rats but not in

CARKO/PXR-knockout rats, thus demonstrating that a CAR/PXR-mediated mechanism was responsible for the liver tumor induction of benfluralin. Additional in vitro studies in cultured hepatocytes from human donors which were treated with benfluralin did not show a proliferation. These results demonstrate that the rodent-specific MOA of CAR/PXR-mediated liver tumor induction by benfluralin is not relevant to humans.
Therefore, overall it could be demonstrated that a CAR/PXR-mediated MOA which is rodent-specific is responsible for the liver tumor induction in the long-term studies in rats and mice.

Thyroid tumor MOA

In a 13-week study in male and female F344 rats the thyroid tumor MOA was investigated. The objective of this study was to investigate if a 13-week administration via diet at a dose-level higher than the carcinogenic dose in rats of 10000 ppm would lead to a decrease of T4 and corresponding increase of TSH levels and to increased liver and thyroid weights. Significantly lower mean T4 concentrations after 4 and 13 weeks of treatment were seen and TSH levels were increased by factors 1.5 up to 2.8. T3 hormone levels were not decreased but slightly increased. Increased liver weights in treated animals were noted in both sexes and correlated with a centrilobular or diffuse hepatocellular hypertrophy in both sexes. Significantly increased thyroid weights were noted in males with a similar trend in females which correlated with the minimal to slight follicular cell hypertrophy/hyperplasia noted in the thyroid gland. The typical decrease of T4 and corresponding increase of TSH was also observed in a male and female pubertal assay.
In order to investigate whether a direct effect on the thyroid could have been involved, the potential of benfluralin to inhibit the Thyroid Peroxidase (TPO) enzyme was investigated in rat thyroid microsomes. TPO was inhibited 76% by positive control 6-N-Propyl-2-Thiouracil (PTU) with an IC50 of 0.79µM. Benfluralin did not show any inhibition of TPO activity up to its highest soluble concentration in incubation medium, 100µM so that this MOA can be excluded.
According to a publication about testing of many compounds, including benfluralin on sodium iodide symporter (NIS) inhibition, benfluralin was negative in this test so that also based on this result no evidence of a direct thyroid effect was detected (Supporting information to Wang et al, 2018).
Altogether these results observed in the rats exclude a direct effect on the thyroid, but support the proposed liver enzyme-mediated mechanism via increased T4 metabolism with subsequently lower T4 levels and corresponding increase of TSH levels which in rodents after lifetime exposure leads to follicular cell hypertrophy/hyperplasia and finally to thyroid tumors. The rodent specificity was supported by an absence of thyroid effects in dogs and by the negative outcome of an AMA. This rodent-specific MOA is known to be of no relevance to humans.

A summary of the key events and assessment criteria to assess the liver and thyroid tumor MOA is given in the following chapters and tables 4 and 5.

4.1 Liver tumors

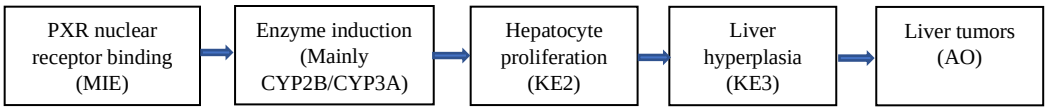
Postulated MOA

The described MOA studies support a liver enzyme-mediated liver tumor MOA which is not relevant to humans. The main key event is the activation of CYP2B/CYP3A which leads to increased liver cell proliferation and via hyperplasia after lifetime exposure to liver tumors. Based on the experience with

phenobarbital and other liver enzyme-inducing compounds this MOA is rodent-specific and not relevant to humans.

Key events of liver tumor MOA

In rodents the cascade of key events after benfluralin treatment is starting with the activation of PXR as molecular initiating event (MIE) which subsequently leads to an increased activation of CYP2B/CYP3A enzymes, followed by cell proliferation with the eventual consequence of hyperplasia and liver tumor formation after lifetime treatment. The key events are summarized as follows:



The key events, PXR nuclear receptor binding (MIE), liver enzyme induction (mainly CYP2B/CYP3A) (KE1) and hepatocyte proliferation (KE2) were observed in the described MOA studies and hyperplasia (KE3) and liver tumors (AO) in the rat and mouse long-term studies.

Concordance of dose-response relationship

The MOA studies demonstrated a clear dose-response relationship of the key events. In this regard the first key event was liver enzyme induction, as measured by increased hepatic phase I enzyme induction of CYP2B/CYP3A at 5000 ppm in F344 rats ([redacted] 2020) and in ex vivo measurements in rats which were treated with 5000 ppm. Hepatocyte proliferation was seen at dose levels inducing enzyme induction ([redacted] 2010). A dose concordance was also obvious in the short-term studies in rats and mice in which liver weights were increased and histopathological findings, like hypertrophy occurred, as well as in the long-term studies in rats and mice. In a subchronic rat study, liver weights were increased from 250 ppm on and hepatocellular hypertrophy as indication of liver enzyme induction was also observed from 250 ppm on and thus at doses which are below the tumor-inducing doses of 2500 and 5000 ppm. In the rat long-term study with doses of 0, 100, 2500 and 5000 ppm, liver weights were increased from 2500 ppm on and centrilobular /diffuse hypertrophy as evidence of liver enzyme induction from 100 ppm on and thus at lower doses than the doses of 2500 and 5000 ppm at which higher liver tumor incidences were noted.

In a subchronic mouse study liver weight increase and hepatocellular hypertrophy which are indicative of liver enzyme induction were seen from 1000 ppm on which is thus below the dose of 1500 ppm at which increase liver tumor incidences were noted. The incidences of liver adenomas and carcinomas showed also a dose concordance from 1500 ppm on.

Temporal relationship

It is obvious from the MOA data that early key events occur before the formation of liver tumors. Thus, activation of the nuclear CAR/PXR receptors which are the most important key events in this MOA and liver enzyme induction occur in the studies very early as observed in the 14-day study. Hepatocellular hypertrophy was also seen within 14 days. In the subchronic toxicity studies in rats and mice hepatocellular hypertrophy was seen at the end of the 90-day studies. Neoplastic liver findings were only seen in the long-term studies.

The temporal relationship for the liver tumor MOA of benfluralin is therefore identical to that of the MOA of phenobarbital.

Strength, consistency and specificity of association of key events and tumor response

The early key events and subsequent events followed the dose and time concordance of the postulated MOA. The MOA studies showed that the liver enzyme induction and PXR receptor activation as the first key event were observed at very early time points as start of the MOA. Indirect measures of these events like liver weight increase and/or liver cell hypertrophy occurred at the same and later time-points.

Biological plausibility

For non-genotoxic agents, an increase in cell proliferation is the common process leading to the development of tumors in general. The similarity to the known phenobarbital-type MOA which leads to increased liver cell proliferation via increased liver enzyme induction and finally to liver tumors, gives further scientific plausibility to the liver tumor MOA of benfluralin. Since the phenobarbital-type liver tumor MOA was intensively investigated and published it can be used as an example. Phenobarbital leads in rodents to an activation of mainly CAR and CYP2B induction which is associated with liver weight increases, hepatocellular hypertrophy, proliferation of smooth endoplasmatic reticulum and cell proliferation as demonstrated by BrdU labelling and with the eventual liver tumor formation. In humans, cell proliferation and tumors are not observed. The importance of the nuclear receptor activation as the first key event in this cascade was proven in studies with phenobarbital in CAR knockout rat hepatocytes/PXR in which no proliferation occurred. The key events after benfluralin are similar to the ones described for phenobarbital, and studies in CARKO/PXRKO animals demonstrated that the PXR effects are also a prerequisite for the benfluralin MOA.
Therefore, it can be concluded that the described MOA for liver tumor induction by benfluralin and the non-relevance for humans are well-supported.

Other possible MOAs

Genotoxicity was confidently excluded. In a 14-day study a MOA via PPAR-α could be excluded (Geter, 2020). There was no evidence of hepatocellular degeneration and necrosis, thus no evidence for a cell death and regenerative proliferation MoA.

An overview is provided in the following table.

Table 4: Alternative Modes of Action for Induction of Liver Tumors in Rodents and Reason(s) for their Exclusion for Benfluralin

Alternative MOA	Reason for exclusion
Genotoxicity	The genotoxicity studies did not indicate a genotoxic potential of benfluralin
Peroxisome proliferator	A MOA via PPAR-α could be excluded (Geter, 2020)
Enzyme induction (aryl hydrocarbon receptor [AhR]-mediated)	CAR/PXR knockout abolishes proliferation in vitro, making a relevant role of AhR highly unlikely

Estrogenic stimulation	An assessment of the endocrine potential of benfluralin by EFSA did not reveal any EAS potential, so that an estrogenic stimulation effect as possible MOA can be confidently excluded
Statins	Benfluralin does not belong to the chemical class of statins and has not such effects, thus liver tumor MOA like that of statins can be excluded
Cytotoxicity	There was no evidence of hepatocellular degeneration and necrosis, thus no evidence for a cell death and regenerative proliferation MoA.
Infection	In the long-term toxicology studies, no evidence of an infection as cause of the observed tumors was obvious
Iron/copper overload	Histopathologically no evidence of an iron or copper overload was seen in the toxicology studies
Increased apoptosis	Histopathologically no evidence of an increased apoptosis was detected in the toxicology studies

Uncertainties, inconsistencies, and possible data gaps

With regard to the proposed liver tumor MOA, no inconsistencies are obvious since the IPCS framework requirements are fulfilled. There is clear concordance between dose, temporality and the expected sequence of events for tumorigenicity of benfluralin as non-genotoxic compound in the liver of rodents. There are only a few data gaps of mainly technical or experimental nature, like for example determination of hepatocellular proliferation in vivo. Since liver proliferation determinations in the in vitro models clearly showed non-relevance to humans, additional vertebrate studies for this purpose do not seem to be justified.

Assessment of the proposed liver tumor MOA and conclusion of human relevance

This postulated MOA has a high degree of reliability, since there is a very good concordance between dose, temporality, and the expected sequence of events for tumorigenicity in the rat and mouse liver. Further support of high reliability of this MOA comes from the good agreement with the MOA of phenobarbital which showed the same concordance between dose, temporality, and the expected sequence of events for its tumorigenic effect in the rodent liver. A large literature basis for this MOA via CAR/PXR activation leading to increased liver cell proliferation and eventually to tumors exists. This rodent-specific liver tumor MOA is not relevant to humans, based on published scientific research and on the fact that phenobarbital which has the same MOA and is used in human medicine since decades, epidemiologically did not show any effect on liver tumors in humans. Another example is momfluorothrin which caused liver tumors in rats by CAR activation and which with reference to the MOA of phenobarbital was regarded as non-relevant to humans by RAC and thus was not classified by the RAC.
Therefore, this rodent-specific liver tumor MOA is not relevant to humans, based on broad published scientific research and on the fact that phenobarbital is used in human medicine since decades and epidemiologically did not show any effect on human liver tumor incidences (Holsapple, 2006).

Table 5: Concordance table liver tumor MOA

Key events	• In rodents the cascade of key events after benfluralin treatment is starting with the activation of CAR/PXR as molecular initiating event (MIE) • followed by an increased activation of CYP2B/CYP3A enzymes, followed by cell proliferation • eventual consequence of hyperplasia and liver tumor formation after lifetime treatment
Concordance of dose-response relationship	• MOA studies demonstrated a clear dose-response relationship of the key events. • First key event was liver enzyme induction, as measured by increased hepatic phase I enzyme induction of CYP2B/CYP3A at 5000 ppm in F344 rats and in ex vivo measurements in rats • Dose concordance was also obvious in the short-term studies in rats and mice in which liver weights were increased and histopathological findings, like hypertrophy occurred dose-dependently • In a subchronic rat study, liver weights were increased from 250 ppm on and hepatocellular hypertrophy as indication of liver enzyme induction was also observed from 250 ppm on and thus at doses which are below the tumor-inducing doses of 2500 and 5000 ppm • In the long-term studies in rats and mice, dose concordance was also seen regarding liver weight increases and histopathological findings; in the rat long-term study with doses of 0, 100, 2500 and 5000 ppm, liver weights were increased from 2500 ppm on and centrilobular /diffuse hypertrophy as evidence of liver enzyme induction from 100 ppm on and thus at lower doses than the doses of 2500 and 5000 ppm at which higher liver tumor incidences were noted • Hepatocellular proliferation was induced dose-dependently in wild-type rats and mice, while not observed in hepatocytes from CAR/PXR knockout rats or humans. • Incidences of liver adenomas and carcinomas showed also a dose concordance from 2500 ppm on
Temporal association	• MOA data show that early key events occur before the formation of liver tumors • Activation of the nuclear CAR/PXR receptors which is the most important key event in this MOA and liver enzyme induction occurred already in the 14-day MOA study • Non-neoplastic and neoplastic liver findings were only seen in the long-term studies • Based on the aforementioned associations the temporal relationship for the liver tumor MOA of benfluralin is similar to that of the MOA of phenobarbital and momfluorothrin
Strength, consistency and specificity of association of tumor response with key events	• The early key events and subsequent events followed the dose and time concordance of the postulated MOA, consistently • MOA studies show that the liver enzyme induction and CAR/PXR receptor activation as the first key event were observed at very early time points as start of the MOA • Indirect measures of these events like liver weight increase and/or liver cell hypertrophy occurred at the same and later time-points • Therefore, the association of the liver tumors with the key events was consistently and specifically obvious in all studies
Biological plausibility and coherence	• For non-genotoxic agents, an increase in cell proliferation is the common process leading to the development of tumors • Similarity to the known phenobarbital-type MOA which leads to increased liver cell proliferation via increased liver enzyme induction and finally to liver tumors, gives further scientific plausibility to the liver tumor MOA of benfluralin • Since the phenobarbital-type liver tumor MOA was intensively investigated and published it can be used as an example for the proposed liver tumor MOA of benfluralin • Key events after benfluralin are similar to the ones described for phenobarbital, and studies in CARKO/PXRKO rat hepatocytes demonstrated that the CAR/PXR effects

	are also a prerequisite for the benfluralin MOA • Therefore, it is concluded that the described MOA for liver tumor induction by benfluralin and the non-relevance for humans are well-supported
Other possible MOAs	• Genotoxicity could be confidently excluded • In a 14-day study ([redacted], 2020) a MOA via PPAR-α could be excluded • The absence of a proliferative response in CARKO/PXRKO rat hepatocytes demonstrates that other nuclear receptor activities besides CAR and PXR are irrelevant for Benfluralin
Uncertainties, inconsistencies and data gaps	• With regard to the proposed liver tumor MOA, no inconsistencies are obvious since the IPCS framework requirements are fulfilled • Clear concordance could be demonstrated between dose, temporality and the expected sequence of events for tumorigenicity of benfluralin as non-genotoxic compound in the liver of rodents • Only a few data gaps of mainly technical or experimental nature, e.g. an evaluation of proliferation in vivo would be supportive, but the results of such additional examinations would most likely not change the MOA.
Assessment of postulated mode of action	• High degree of reliability for this postulated MOA since there is a very good concordance between dose, temporality, and the expected sequence of events for tumorigenicity in the rat and mouse liver • Further support of high reliability of this MOA comes from the good agreement with the MOA of phenobarbital, and the large literature basis for this MOA • This rodent-specific liver tumor MOA is not relevant to humans, based on published scientific research and on the fact that phenobarbital which has the same MOA and is used in human medicine since decades, epidemiologically did not show any effect on liver tumor incidences in humans

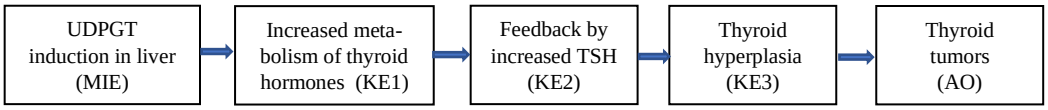
4.2 Thyroid tumors

Postulated MOA

In a 13-week study in male and female F344 rats it could be demonstrated that a 13-week oral administration via diet at a dose-level higher than the carcinogenic dose in rats leads to a decrease of T4 and to a corresponding increase of TSH levels, furthermore to increased liver and thyroid weights. At the same dose at which thyroid effects occurred also liver effects, particularly increased liver weights in treated animals were noted in both sexes and correlated with a centrilobular or diffuse hepatocellular hypertrophy in both sexes. This was not only seen in the MOA studies, but also in the pubertal assays in male and female rats. Therefore, also the thyroid effects are caused by liver enzyme induction, especially of UDPGT which in rodents leads to an increased thyroid hormone metabolism with subsequently lower T4 levels and increased TSH feedback; subsequently following events consist of hypertrophy, hyperplasia and eventually thyroid tumor induction. This is a rodent-specific MOA since no thyroid effects were seen in in dogs or an AMA. A direct effect on the thyroid via inhibition of thyroid peroxidase or the sodium-iodide-transporter could be excluded.
Altogether these results observed in the rats exclude a direct effect on the thyroid, but support the proposed liver enzyme-mediated mechanism via increased T4 metabolism with subsequently lower T4 levels and corresponding increase of TSH levels which after lifetime exposure of rodents lead to follicular cell hypertrophy/hyperplasia and finally to thyroid tumors. This MOA is known to be of no relevance to humans. A summary of the key events and assessment criteria to assess this thyroid tumor MOA is given in the following chapters.

Key events of thyroid MOA

An increased incidence of thyroid tumors was only seen in rats. The key events are summarized as follows:



The key events, UDPGT induction in liver (MIE), increased metabolism of thyroid hormones (KE1) and feedback by increased TSH (KE2) were observed in the described MOA studies and thyroid hyperplasia and thyroid tumors in the rat long-term study.

Concordance of Dose-response relationship

The effects on thyroids occurred only at doses which caused liver effects and it is postulated that the thyroid effects are secondary to the observed liver effects. Especially liver enzyme induction is known to cause an increased metabolism and excretion of thyroid hormones which leads to a feedback mechanism of a TSH release with higher TSH levels which after exposure to high doses over longer time periods leads to a stimulation of the thyroids. This relationship between the thyroid and the liver effects was demonstrated in all MOA studies in rats and in the short- and long-term rat studies in which thyroid effects only occurred at doses at which also liver effects were seen.

Temporal relationship

Since there is a causal relationship between the thyroid and the liver effects also the temporal relationship is comparable to that of the liver MOA. Thus, the first effect consists of an early liver enzyme induction which includes induction of UDPGT as molecular initiating event (MIE) as was demonstrated in a MOA study in rats. This demonstrated that the thyroid effects can be explained as a consequence of liver enzyme induction which is known to cause an increased metabolism and excretion of thyroid hormones, leading to lower T4 levels and to a feedback mechanism of a TSH release with higher TSH levels which over longer time periods leads to a stimulation of the thyroids and eventually after lifetime exposure of rodents to higher doses can induce thyroid tumors. This relationship between the thyroid and the liver effects was demonstrated in MOA studies in rats and in the short- and long-term rat studies in which thyroid effects only occurred at time-points at which also liver effects were seen.

Strength, consistency and specificity of association of key events and tumor response

Like for the liver tumor MOA, also for the thyroid MOA the early key events and subsequent events followed the dose and time concordance of the postulated MOA. Liver enzyme induction, including especially UDPGT induction, as the first key event were observed at very early time points and subsequent events, like thyroid weight effects and histopathological findings in the thyroid which indicate a thyroid stimulation by TSH were observed in the rat studies. Thyroid effects were only observed at doses and time-points at which also liver effects occurred which supports the strength, consistency and specificity of this MOA.

Biological plausibility

For non-genotoxic agents, an increase in cell proliferation is the common process leading to the development of tumors in general. The similarity to other published liver enzyme inducers, like

phenobarbital, sulfonamides and other compounds, which lead to an activation of UDPGT and subsequently to an increased metabolic clearance of thyroid hormones and as feedback to an increased TSH release with lifetime stimulation of the thyroid and eventually increased chance of tumor induction (in rodents), gives further scientific support to the proposed thyroid tumor MOA of benfluralin. No comparable effects were seen in dogs, for which a substantial study program exists. This MOA is not regarded as relevant to humans. The proposed MOA for thyroid tumor induction by benfluralin and the non-relevance for humans are well-supported.

Other possible MOAs

In order to investigate whether a direct effect on the thyroid could have been involved, the potential of benfluralin to inhibit the Thyroid Peroxidase (TPO) enzyme was investigated in rat thyroid microsomes. TPO was inhibited by the positive control 6-N-Propyl-2-Thiouracil (PTU) in this study, demonstrating the ability of the study to detect TPO inhibitors. In this study, benfluralin did not show any inhibition of TPO activity up to its highest soluble concentration in incubation medium of 100µM so that this MOA can be excluded.
In a publication about testing of many compounds on sodium iodide symporter (NIS) inhibition (Supporting information to Wang et al, 2018), also benfluralin was tested. Benfluralin proved to be negative in this test so that also based on this result no evidence of a direct thyroid effect was detected. Altogether these results observed in the rats exclude a direct effect on the thyroid, but support the proposed liver enzyme-mediated mechanism via induction of UDPGT and increased T4 metabolism with subsequently lower T4 levels and corresponding increase of TSH levels which via continuous thyroid stimulation lead to follicular cell hypertrophy/hyperplasia and finally to thyroid tumors after lifetime exposure of rodents to high doses. This MOA is known to be of no relevance to humans.

Uncertainties, inconsistencies, and possible data gaps

With regard to the proposed thyroid tumor MOA, no inconsistencies are obvious since the IPCS framework requirements are fulfilled. There is clear concordance between dose, temporality and the expected sequence of events for tumorigenicity of benfluralin as non-genotoxic compound in the thyroid of rodents.
There are no obvious data gaps, and a direct effect on the thyroid could be excluded. Although UDPGT data are available, determination of the UDPGT in a subacute study in rats could be considered, but the results of such additional examinations would most likely not change the MOA.

Assessment of the proposed thyroid tumor MOA and conclusion of human relevance

The thyroid tumor MOA has a high reliability since a very good concordance between dose, temporality, and the expected sequence of events for tumorigenicity in the thyroid and agreement with a broad database of published work in this area was established. Further support of high reliability of this MOA exists due to very good agreement with the thyroid tumor MOA of phenobarbital and other compounds which showed the same concordance between dose, temporality, and the expected sequence of events for tumor induction in the rodent thyroid. A large literature basis for this MOA via UDPGT induction leading to increased thyroid hormone clearance and TSH feedback with lifetime thyroid stimulation by TSH which eventually leads to thyroid tumors exists. This rodent-specific thyroid tumor MOA is not relevant to humans, based on published scientific research and on the fact that phenobarbital which has the same

MOA and is used in human medicine since decades, epidemiologically did not show any effect on thyroid tumors in humans.
There is no relevance of such findings to humans due to differences in thyroid physiology between rodents and humans (Capen, 1997; Boobis et al 2006, Curran, 1991). There are many reasons for the difference in response between rodents and humans; rodents have an enhanced thyroid hormone elimination as compared to humans, thyroxin binding globulin is a major plasma protein in humans (which acts as a buffer), but not in rodents. As a consequence, the concentration of unbound T4 is greater in rodents than humans, resulting in greater susceptibility to metabolism and excretion and compensatory increase in thyroid follicular cell turnover. According to Capen, 1997, "there is no convincing evidence that humans treated with drugs or exposed to chemicals that induce hepatic microsomal enzymes are at increased risk for the development of thyroid cancer".

Table 6: Concordance table for thyroid tumor MOA

Key events	• In rodents the cascade of key events after benfluralin treatment is starting with the activation of UDPGT as molecular initiating event (MIE) • followed by increased metabolism of thyroid hormones (KE1), feedback by increased TSH (KE2) as observed in the described MOA studies • followed by thyroid hyperplasia and thyroid tumors after lifetime treatment
Concordance of dose-response relationship	• Thyroid effects occurred only at doses which caused liver effects so that the thyroid effects are regarded as secondary to the observed liver effects • Liver enzyme induction, specially of UDPGT is known to cause an increased metabolism and excretion of thyroid hormones which leads to a feedback mechanism of a TSH release with higher TSH levels • Lifetime increase of TSH levels are known to lead to a stimulation of the thyroids • A good dose- and time-relationship between the thyroid and the liver effects was demonstrated in all MOA studies in rats and in the short- and long-term rat studies
Temporal association	• First effect consists of an early liver enzyme induction which includes induction of UDPGT as molecular initiating event (MIE) as demonstrated in a MOA study in rats • This demonstrated that the thyroid effects can be explained as a consequence of liver enzyme induction as first event • Increased activity of UDPGT is known to cause an increased metabolism and excretion of thyroid hormones, leading to lower T4 levels and to a feedback mechanism of a TSH release with higher TSH levels following the first events • A decrease of T4 and an increase of TSH was also seen in pubertal assays in male and female rats • Increased TSH levels over the whole lifetime of rodents leads to a stimulation of the thyroid tissues and eventually to thyroid tumors • The aforementioned relationship between the thyroid and the liver effects was demonstrated in MOA studies and in the short- and long-term rat studies in rats
Strength, consistency and specificity of association of tumor response with key events	• Like for the liver tumor MOA, also for the thyroid MOA the early key events and subsequent events followed the dose and time concordance of the postulated MOA and thus demonstrate the strength and consistency of the proposed MOA • Liver enzyme induction, including especially UDPGT induction, as the first key event were observed at very early time points and subsequent events, like thyroid weight effects and histopathological findings in the thyroid followed later • Thyroid effects were only observed at doses and time-points at which also liver effects occurred which supports the strength, consistency and specificity of this liver-mediated thyroid tumor MOA. • No comparable effects were observed in dogs
Biological plausibility and coherence	• For non-genotoxic agents, an increase in cell proliferation is the common process leading to the development of tumors in general • Similarity to other published liver enzyme inducers, like phenobarbital, sulfonamides

	and other compounds, with a similar MOA as proposed for benfluralin supports biological plausibility and gives scientific support to the proposed thyroid tumor MOA of benfluralin • Similarity to the thyroid tumor MOA of fluopyram which caused thyroid tumors in mice via UDPGT induction and which was accepted as non-relevant to humans by RAC and did not lead to classification based on the thyroid tumors • Therefore, it is concluded that the biological plausibility of the proposed MOA for thyroid tumor induction by benfluralin and the non-relevance for humans are well-supported by the data and literature
Other possible MOAs	• An investigation of inhibition of the Thyroid Peroxidase (TPO) enzyme by benfluralin did not show any inhibition of TPO activity by benfluralin so that this MOA can be excluded • Benfluralin which was tested among other chemicals on sodium iodide symporter (NIS) inhibition was negative in the test so that this MOA can also be excluded • Altogether a direct effect on the thyroid can be excluded, the data support the proposed indirect phenobarbital-like liver enzyme-mediated mechanism of thyroid tumor induction
Uncertainties, inconsistencies and data gaps	• With regard to the proposed thyroid tumor MOA, no inconsistencies are obvious since the IPCS framework requirements are fulfilled • There is clear concordance between dose, temporality and the expected sequence of events for tumorigenicity of benfluralin as non-genotoxic compound in the thyroid of rodents • A direct effect on the thyroid could be excluded • Although UDPGT data are available, determination of the UDPGT in the 90-day study in rats could be considered, but the results of such additional examinations would most likely not change the MOA
Assessment of postulated mode of action	• High degree of reliability for this postulated MOA since there is a very good concordance between dose, temporality, and the expected sequence of events for tumorigenicity in the rat thyroid • Further support of high reliability of this MOA due to very good agreement with the thyroid tumor MOA of phenobarbital and other compounds which showed the same concordance between dose, temporality, and the expected sequence of events for tumor induction in the rodent thyroid • A large literature basis for this MOA via UDPGT induction leading to increased thyroid hormone clearance and TSH feedback with lifetime thyroid stimulation by TSH which eventually leads to thyroid tumors exists for many other compounds • The rodent specificity was demonstrated by an absence of thyroid effects in the AMA • This rodent-specific thyroid tumor MOA is not relevant to humans, based on published scientific research and on the fact that phenobarbital which has the same MOA and is used in human medicine since decades, epidemiologically did not show any effect on thyroid tumors in humans • There is furthermore no relevance of such findings to humans due to differences in thyroid physiology between rodents and humans (Capen, 1997; Boobis et al 2006, Curran, 1991). There are differences in response between rodents and humans which renders rodents more sensitive to changes than humans • According to Capen, 1997, “there is no convincing evidence that humans treated with drugs or exposed to chemicals that induce hepatic microsomal enzymes are at increased risk for the development of thyroid cancer”.

5. Overall Summary and Conclusions

Based on this evaluation, it is concluded that the liver MOA via a liver enzyme induction is not relevant to humans since the MOA is similar to that of phenobarbital which causes liver tumors in rodents, but not in humans. Another example supporting this conclusion is fluopyram which caused thyroid tumors via UDPGT induction and subsequently increased TSH levels leading to lifetime thyroid stimulation. This compound was assessed by RAC which concluded that this thyroid tumor MOA is not relevant to humans and therefore was not classified based on the thyroid tumors.
With regard to the thyroid tumors, a direct effect on the thyroid can be excluded, and also for this tumor a liver enzyme-mediated mechanism was supported which via increased T4 metabolism with corresponding increase of TSH levels which leads to follicular cell hypertrophy/hyperplasia and finally to thyroid tumors. This MOA is known to be of no relevance to humans.

Therefore, it is concluded that from both tumor types, the liver and the thyroid tumors as noted in the long-term studies in rodents no risk for humans is expected.

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Appendix 1

In vitro studies

Methods:

The studies in the different cultured hepatocytes were conducted according to the general methods as described in the following method description.

Justification of Test System

Test Item-mediated hepatocellular effects observed *in vivo* such as increased enzyme activities and proliferation can be modelled *in vitro* using primary rodent hepatocytes in culture.

Characterization of Test System

Hepatocytes were isolated from male F344 rats by means of a two-step collagenase perfusion according to Mitchell *et at,* (1984) and following SOP CLS4_ROD_013. Details were described under study number CLS4_0001_0011. The average viability of cell preparations was greater than 80%.

Hepatocyte Culture

Primary monolayer cultures of hepatocytes were established in 96-well or 6-well plates. In 96-well plate cultures the outside wells were not used but filled with sterile phosphate buffered saline (to prevent edge effects caused by evaporation of culture media). Freshly isolated hepatocytes were cultured in Leibowitz CL15 medium (SOP: CLS4_HEP_002) for approximately 4 hours to allow adherence to vessels in a humidified incubator at 37 °C under atmospheric air. The medium was then replaced with fresh Leibowitz CL15 medium containing PB at 1 concentration (1000 µM), benfluralin at 4 concentrations, (3, 10, 30 and 100 µM) or vehicle control [0.1% v/v dimethylsulfoxide (DMSO)] and cultured in a humidified incubator at 37 °C under air. In addition to hepatocytes exposed to Test Item or PB, 5 replicates in 6-well plates were exposed to EGF (25 ng/ml) as a positive control for induction of replicative DNA synthesis (S-phase). The Leibowitz CL15 media contained foetal bovine serum, tryptose phosphate broth, penicillin/streptomycin, insulin, hydrocortisone 21-hemisuccinate, L-glutamine and gentamycin.
Benfluralin was formulated in DMSO and administered such that the final DMSO concentration in all cultures was 0.1% (v/v) (1 µl DMSO/mL medium). Benfluralin was made fresh each day. The media, including test item or positive control, was replenished daily for a further 3 days in CL15 media containing 5-bromo-2′-deoxyuridine (BrdU) (10 µM). There were 6 replicates for each concentration in 96-well plates for cell toxicity (ATP) measurements, 5 replicates for each concentration in 6-well plates for S-phase analysis and 3 replicates for each concentration in 6-well plates for potential future mRNA analysis. ATP assays were carried out at approximately 92 hours post seeding with daily exposure to test item.

Cytotoxicity (ATP)

Cytotoxicity was assessed following approximately 92 hours of culture by measuring ATP concentrations by luminometry according to the SOP (CLS4_TIC_004) and analysed using a Hidex Sense Microplate reader with Hidex Sense PlateReader Software version 0.5.55.0. The bioluminescent determination of ATP released from viable cells was carried out using an assay kit supplied by Promega (CellTitre-Glo luminescent cell viability assay) according to manufacturer’s instructions. Results were expressed as a percentage of the amount of ATP released from the control cells.

Replicative DNA Synthesis (S-Phase)

S-phase was determined immunocytochemically by determining the incorporation of BrdU into hepatocyte nuclei over the last 3 days of culture. Immunostaining was performed after fixation at approximately 96 hours using a mouse monoclonal anti-Bromodeoxyuridine Clone Bu20a primary antibody (Agilent, M0744) and a polyclonal rabbit anti-mouse immunoglobulins/horse radish peroxidase secondary antibody (Agilent, P0260). The number of hepatocytes in S-phase was assessed by manual counting and recorded as the labelling index [(no. of labelled hepatocytes /total no. of hepatocytes) x 100]. Cells were counted under x20 magnification of a light microscope. Four independent fields of view per well of a 6-well plate were counted; this was carried out in 5 wells per treatment, providing 20 fields of view per condition in total. At least 300 hepatocytes were counted per field of view. Cells which were not deemed morphologically normal at any concentration were not counted.

Results

Induction of DNA synthesis in cultured male Sprague-Dawley rat hepatocytes (CLS4_0014_0011)

Treatment with benfluralin stimulated cell proliferation in cultures of primary hepatocytes isolated from Sprague Dawley rats in a dose dependent manner to a maximum of 1.8-fold at 300 µM.

Treatment with benfluralin led to a statistically significant reduction in ATP levels after treatment with benfluralin at 300 µM. This is indicative of toxicity and shows that the system was tested to the highest possible concentration. Presence of precipitated compound was observed in media containing concentrations of 100 and 300 µM benfluralin daily. Statistically significant increases in ATP levels were observed at 10, 30, 100 µM benfluralin and 1mM PB. This is indicative of a normal adaptive response. Treatment with EGF (25 ng/mL) resulted in a statistically significant increase in S-Phase of 3.4-fold of control (Figure 2 and Table 2), which indicates that hepatocytes from Sprague Dawley rats were able to proliferate following exposure to a proliferative stimulus and thus demonstrated their suitability for use in this investigation. Treatment with the positive control, PB, also resulted in an increase in S-Phase of 1.4-fold of control.

Table 4: Effect of benfluralin, PB or EGF on S-Phase in primary hepatocytes prepared from male Sprague Dawley rats

Test Item & Concentration	S-Phase (labelling index)
Vehicle control (0.1% [v/v] DMSO)	6.04 ± 0.59 (100.00 ± 9.8)
PB 1 mM	8.39 ± 0.57 (139.0 ± 9.4) ***
Benfluralin 10 µM	6.42 ± 0.14 (106.4 ± 2.3)
Benfluralin 30 µM	8.72 ± 0.61 (144.5 ± 10.1) ***
Benfluralin	10.77 ± 0.33

100 µM	(178.4 ± 5.5) ***
Benfluralin 300 µM	11.04 ± 0.31 (182.8 ± 5.2) ***
EGF 25 ng/mL	20.40 ± 1.27 (338.0 ± 21.1) ***

Labelling index (%) was used to determine S-Phase for rat hepatocytes treated with benfluralin, PB, EGF or vehicle control (DMSO). Values are Mean ± SD. Values in parenthesis are mean % control ± SD; n = 5 per group. Statistical significance between benfluralin and DMSO control was tested using a one-way ANOVA followed by a Dunnett's multiple comparison test. A comparison between DMSO control and PB or EGF treated hepatocytes was performed using a Student' t-test. *** statistically different from control p< 0.001.

Induction of DNA synthesis in cultured male F344 rat hepatocytes (CLS4_0014_0010)

Treatment with benfluralin up to 30 µM did not result in any statistically significant decreases in intracellular ATP levels when compared to control (Figure 1 and Table 1). However, statistically significant reduction in ATP levels was observed after treatment with benfluralin (100 µM) (69% of control). This is indicative of toxicity and shows that the system was tested to the highest possible concentration. Presence of precipitated compound was observed in media containing concentrations of 100 µM benfluralin, daily. Treatment of hepatocytes with PB resulted in a small but statistically significant reduction in ATP levels to 89% of control, however, this was not deemed to be biologically significant.

Treatment with EGF (25 ng/mL) resulted in a statistically significant increase in S-Phase of 4.8-fold of control (Figure 2 and Table 2). This indicates that hepatocytes from F344 rats could proliferate following exposure to a mitogen and demonstrating their suitability for use in this investigation. Treatment with the positive control, PB, also resulted in an increase in S-Phase of 1.3-fold of control. Treatment with benfluralin stimulated cell proliferation in cultures of primary hepatocytes isolated from F344 rats at 10, 30 and 100 µM with a maximum induction of 1.4-fold (10 µM).

Table 5: Effect of benfluralin, PB or EGF on S-Phase in primary hepatocytes prepared from male F344 rats

Test Item & Concentration	S-Phase (labelling index)
Vehicle control (0.1% [v/v] DMSO)	5.46 ± 0.31 (100.00 ± 5.7)
PB 1 mM	7.08 ± 0.06 (129.7 ± 1.1) ***
Benfluralin 3 µM	5.86 ± 0.43 (107.4 ± 8.0)

Benfluralin 10 µM	7.72 ± 0.37 (141.5 ± 6.8) ***
Benfluralin 30 µM	6.15 ± 0.28 (112.6 ± 5.1) *
Benfluralin 100 µM	6.21 ± 0.15 (113.9 ± 2.8) **
EGF 25 ng/mL	26.10 ± 1.84 (478.4 ± 33.7) ***

Labelling index (%) was used to determine S-Phase for rat hepatocytes treated with benfluralin, PB, EGF or vehicle control (DMSO). Values are Mean ± SD. Values in parenthesis are mean % control ± SD; n = 5 per group. Statistical significance between benfluralin and DMSO control was tested using a one way ANOVA followed by a Dunnett's multiple comparison test. A comparison between DMSO control and PB or EGF treated hepatocytes was performed using a Student' t-test. * statistically different from control $p<0.05$; ** $p< 0.01$; *** $p< 0.001$.

Induction of DNA synthesis in cultured male CARKO/PXRKO rat hepatocytes (CLS4_0014_0012)

The aim of this study was to investigate the potential of benfluralin to stimulate cell proliferation (measured as the change in replicative DNA synthesis, hereafter referred to as S-Phase) in cultures of primary hepatocytes isolated from male Sprague Dawley rats nulled for the constitutive androstane receptor and the pregnane X receptor (CARKO/PXRKO rats). In parallel, cytotoxicity was determined by expressing adenosine 5´-triphosphate (ATP) levels in all wells as a function of ATP levels in control samples. Samples were also harvested for potential future mRNA analysis but were not analysed in this study.

Exposure to benfluralin concentrations up to and including 300 µM did not result in any reduction of ATP levels. Higher concentrations of benfluralin could not be tested due to maximum solubility being achieved. Treatment with benfluralin did not stimulate cell proliferation in cultured hepatocytes from CARKO/PXRKO rats.

Table 6: Effect of benfluralin, PB or EGF on S-Phase in primary hepatocytes prepared from male CARKO/PXRKO rats

Test Item & Concentration	S-Phase (labelling index)
Vehicle control (0.1% [v/v] DMSO)	4.45 ± 0.16 (100.00 ± 3.6)
PB 1 mM	3.82 ± 0.29 (85.9 ± 6.5) **
Benfluralin 10 µM	4.17 ± 0.22 (93.7 ± 4.9)

Benfluralin 30 µM	4.19 ± 0.41 (94.3 ± 9.2)
Benfluralin 100 µM	3.86 ± 0.33 (86.9 ± 7.5) *
Benfluralin 300 µM	3.73 ± 0.22 (84.0 ± 5.0) **
EGF 25 ng/mL	15.43 ± 1.18 (346.9 ± 26.6) ***

Labelling index (%) was used to determine S-Phase for rat hepatocytes treated with benfluralin, PB, EGF or vehicle control Values are Mean ± SD. Values in parenthesis are mean % control ± SD; n = 5 per group. Statistical significance between benfluralin and DMSO control was tested using a one-way ANOVA followed by a Dunnett's multiple comparison test. A comparison between DMSO control and PB or EGF treated hepatocytes was performed using a Student' t-test. Statistically different from control: * ($p < 0.05$); ** ($p<0.01$) and *** ($p<0.001$).

Appendix 2

In vitro studies in hepatocytes of human donors

As part of mode of action studies on the liver tumor potential of benfluralin, in vitro studies in hepatocytes of wildtype and CAR/PXR knockout rats and of human donors (Numbers 385, 8210, 8219, 8239) were performed. From these studies it was concluded that wildtype rat hepatocytes showed proliferation whereas hepatocytes of knockout rats did not.
No proliferation was seen in human hepatocytes which demonstrated thus non-relevance of the liver tumors to humans. This is based on the data from a study in hepatocytes from three human donors which did not show any proliferation in the hepatocytes of donors 8210, 8219 and 8239 at concentrations of up to and including 30 µM.
The results of a test in another donor (no. 385) which was performed separately from the other three donors, showed peaks at 30 and 300 µM and thus no clear concentration-response relationship; furthermore, most individual values of the benfluralin groups were within historical control data ranges of this laboratory. A statistical outlier test with the data of the first donor (385) was not conclusive, however, the data show that they are clearly not in line with the data of the three other donors.
Also the study report author concluded that "Although treatment of cultured human hepatocytes with benfluralin at 30- and 300 µM did cause statistically significant increases in cell proliferation, no significant increase was seen at the intervening concentration of 100 µM. On the basis of this lack of dose response, the increase in S-phase labelling by benfluralin was not deemed to be a genuine result."

In the test with the first donor 385 the highest concentration of 300 µM proved to be very cytotoxic with viability values below 70-80%; also at 100 µM the viability was below 70-80% in most donors. Even at the lower concentration of 30 µM donor 2 (8210) had 'excessive cytotoxicity' (marked with #) and could not be used for evaluation of proliferation, but since we have 2 donors (8219, 8239) with data at 30 µM, 30 µM seems to be the highest concentration which led to meaningful results and can be used for a sound evaluation of a proliferation potential. Due to this cytotoxicity-concentration relationship the laboratory did not try 100 µM for donor 8239, but only tested up to and including 30 µM. This led to the fact that proliferation values for 100 and 300 µM are only available from one of the 4 donors, i.e. donor 385 which thus makes a statistical evaluation difficult. In addition the data for this donor showed a higher variability at concentrations above 30 µM as compared with lower doses, which indicates a lower reliability of the data above 30 µM. This further seems to indicate that there might have been technical issues with the cell cultures at the higher concentrations so that also from this aspect the data at higher concentrations are not reliable.

Based on these aspects and since the study report author concluded that the increase in S-phase labelling by benfluralin was not deemed to be a genuine result, it is concluded that the use of the data of the first donor 385 for the overall conclusion of the outcome of these tests is inappropriate from a scientific standpoint. Therefore, the data from donor 385 cannot be used for the overall evaluation of these tests.

Appendix 3

13-Week thyroid study by oral route (dietary formulation) in F344 rats [redacted], 2019)

The objective of this study was to demonstrate that a 13-week oral administration (dietary admixture) of benfluralin at a dose-level higher than the carcinogenic dose in rats was able to increase TSH level as well as liver and thyroid weights as previously described.

One group of Fischer 344 rats (10/sex) was administered the test item, benfluralin (batch No. 16/2018), through a dietary admixture at the concentration of 10000 ppm for 13 weeks. Another control group of 10 males and 10 females received the untreated diet only under the same experimental conditions and acted as a control group.

The animals were checked daily for mortality and clinical signs.

Body weight was recorded once pre-test, on the first day of treatment and then at least once a week until the end of the study. Food consumption was recorded weekly.

A functional observation battery test was conducted on Week 12.

Hematology, coagulation and blood biochemistry investigations were performed at the end of the treatment period.

Thyroid hormones measurements were performed on Week 4 and at the end of the treatment period.

On completion of the treatment period, all surviving animals were euthanized and a full macroscopic post mortem examination was performed.

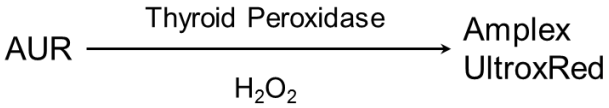
Liver, thyroids with parathyroids and pituitary gland were weighed and preserved for microscopic examination.

Appendix 4

Inhibition of Rat microsomal Thyroid Peroxidase by Benfluralin (Richert et al, 2019)

In order to investigate whether a direct effect on the thyroid could have been involved, the potential of benfluralin to inhibit the thyroid peroxidase (TPO) enzyme was investigated in rat thyroid microsomes.

The thyroid peroxidase activity determination was performed using the substrate fluorogenic Amplex UltraRed (AUR) which is converted to fluorescent Amplex UltroRex by TPO in the presence of excess H_2O_2, according to an SOP in place at KaL-Cell:



In a black 96-well plate 12.5µL of thyroid microsomes (0.0625mg/mL final total protein concentration), 50µL of 0.2M phosphate incubation buffer (pH7.4), 12.5µL of Benfluralin or PTU at increasing concentration or DMSO and 12.5µL of H_2O_2 (300µM; final concentration) were mixed. Incubation was performed in triplicate. Incubation plate was kept at 37°C for 10min in a thermostatically controlled shaker before the reaction was started by addition of 12.5µL AUR (25mM final concentration). After 10min of incubation the fluorescence was read (Ex=540nm; Em=580nm). Blank incubations consisted of the same reaction mix with heat-inactivated microsomes (5min at 85°C).
TPO was inhibited 76% by positive control 6-N-Propyl-2-Thiouracil (PTU) with an IC50 of 0.79µM.
Benfluralin did not show any inhibition of TPO activity up to its highest soluble concentration in incubation medium, 100µM so that this MOA can be excluded.