benthiavalicarb-isopropyl

ADDITIONAL INFORMATION ON ENDOCRINE PROPERTIES

ASSESSMENT OF ENDOCRINE DISRUPTING PROPERTIES AND COMMENTS ON EFSA'S EVALUATION

TABLE OF CONTENTS

1 INTRODUCTION

In accordance with Article 13(3a) of Regulation (EU) No 844/2012, as amended by Regulation (EU) No 2018/1659 in view of the implementation of the scientific criteria for the determination of endocrine disrupting properties introduced by Regulation (EU) No 2018/605, the European Food Safety Authority (EFSA) was able to conclude that the scientific criteria for the determination of endocrine disrupting properties set out in point 3.6.5 of Annex II to Regulation (EC) No 1107/2009 are met. K-I Chemical Europe SA/NV was informed about this conclusion in a letter received on 18 January 2019 that included the minutes of two expert consultations held on 20 November 2018 and 9 January 2019. In the first consultation it was concluded that the thyroid effects in male mice were secondary to liver toxicity. The uterine tumours in female rats were however considered to be likely endocrine-mediated. In order to reach a conclusion it was proposed to reconsider the toxicological data package for assembling lines of evidence in accordance with the methodology described in the *Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009*. The lines of evidence were reported in the Renewal Assessment Report. The evidence provided allowed the scientific assessor of the rapporteur Member State to conclude that benthiavalicarb-isopropyl does not meet the ED criteria. In the second consultation, it was EFSA's opinion that the thoroughly assembled lines of evidence were nonetheless insufficient to support definitive modes of action for the different adverse endocrine adverse effects, *i.e.* uterine adenocarcinoma in female rats and increased incidence of ovarian atrophy and uterine angiectasis in female mice. Therefore, it was concluded that benthiavalicarb-isopropyl satisfies the criteria for a human endocrine disruptor.

In order to address the conclusion that benthiavalicarb meets the scientific criteria of a human endocrine disruptor, additional information is submitted, including (1) an extensive assessment of the potential endocrine disruptor properties and comments on EFSA's peer review meeting minutes (Appendix A); (2) an assessment of the exposure of humans in relation to the endocrine/carcinogenic effect levels (Appendix B) and (3) an evaluation of the agricultural necessity of benthiavalicarb-isopropyl (Appendix C). Considering that benthiavalicarb is one of the first active substances assessed under the new Guidance for the identification of endocrine disruptors in biocides and plant protection products, in particular the argumentation provided in Appendix A should be a basis of a wider consultation amongst Member States allowing a reconsideration of the draft conclusion reached.

2 EXECUTIVE SUMMARY

2.1 Part I: Applicant's extensive assessment of the endocrine disrupting properties

The joint ECHA/EFSA ED guidance has been followed as much as possible for the potential endocrine properties of benthiavalicarb-isopropyl. All available information have been extracted and reported in the Appendix E of the ED GD (see Annex 1 of this document). Then, all lines of evidence have been assembled, assessed, integrated and reported in two tables, one for the E, A, S modalities (Annex 2 of this document) and one for the T modality (Annex 3 of this document). Another table was also provided with some of the main toxic effects regarding general toxicity, mainly on liver and kidney (Annex 4 of this document).

The maximum tolerated doses (MTD) have also been derived following the appropriate definitions from the toxic effects observed in short term and long term repeated dose oral toxicity studies in mice, rats and dogs. According to the ED Guidance potentially endocrine-related adverse effects only observed at excessive toxic dose/concentration (i.e. only observed above the MTD or MTC) should not be considered indicative of endocrine disruption. Although the adverse effects observed at excessive toxicity must not be considered in the assessment of potential endocrine disrupting properties, these effects were taken into account in the assessment in order to dismiss any doubts.

For E, A and S modalities, the major adverse effect observed at excessive toxicity was uterine adenocarcinoma in rats at 5,000 and 10,000 ppm in the carcinogenicity study at 78 and 104 weeks. Known non-hormonal and hormonal mechanisms for uterine adenocarcinoma in rats are (1) genotoxicity, (2) direct estrogenicity, (3) interference with the hormone balance such as by liver enzyme induction and (4) dopamine agonism. Hormonal modes of action for the development of endometrial cancer in the ageing rat can be due to (1) exposure to estrogenic compounds, (2) increase of the E/P-ratio, (3) modulation of estrogen metabolism through induction of CYP1A1, CYP1A2, and CYP1B1 liver enzymes with the production of 2-hydroxyestradiol (2-OHE_2) and 4-hydroxyestradiol (4-OHE_2). These three possibilities have been explored for benthiavalicarb-isopropyl. Firstly, an uterotrophic bioassay has shown that benthiavalicarb-isopropyl is not estrogenic. Secondly, the E/P-ratio has been measured in the carcinogenicity study in rats and in an *in vivo* mechanistic 8-weeks rat study at di-oestrus phase. In both studies the ratio was always consistent with the control groups at all sampling times and all tested doses. Thirdly, possible modulation of estrogen metabolism through activation of AhR was also addressed in an *in vivo* mechanistic study in which the induction of CYP1A1, CYP1A2 and CYP1B1 liver enzymes was not biologically significant. Based on current scientific knowledge on endocrine-mediated endometrial cancers, it is presumed that that the mechanism involved in the development of uterine adenocarcinoma is non-hormone related. It should be noted that uterine adenocarcinoma only developed at excessively toxic doses.

For the T modality, the major adverse effect observed at excessive toxicity was thyroid adenoma and carcinoma in mice. The mode of action was fully elucidated according to the ED GD as both protocol and scientific knowledge were available. The postulated MoA for the induction of thyroid follicular cell tumours in the mouse by benthiavalicarb-isopropyl was verified and involves activation of Car/Pxr hepatic nuclear receptors (KE1) leading to the induction of T4-UDP-GT in the liver (KE2). Enhanced conjugation of T4 and T3 with glucuronic acid increases the excretion of thyroid hormones via the bile with the reduction of T4 and T3 plasma levels as a consequence. Since rodents do not have thyroid hormone binding globulin, the change in plasma levels is instant and is immediately detected by the hypothalamus activating the hypothalamic-pituitary-thyroid (HPT) axis to restore thyroid hormone homeostasis. The hypothalamus produces thyrotropin releasing hormone (TRH) which binds to the TRH receptor in the pituitary increasing the production of thyroid stimulating hormone (TSH) by the basophilic cells in the anterior pituitary (KE3). TSH activates the thyroid to produce more thyroid hormone. Continuous stimulation of the thyroid by TSH leads to thyroid follicular cell hypertrophy and follicular cell proliferation (KE4), which ultimately gives rise to the formation of thyroid tumours (KE5). This mode of action is similar to the mode of action phenobarbital and is not relevant to human.

2.2 Part II: Inconsistencies and oversights noted in EFSA's peer review assessment

Several inconsistencies between EFSA statements and the available dossier were noticed in the minutes of the two expert meetings (PPP Expert meeting 186 and PPP Expert TC 203) during which the potential endocrine disrupting properties of benthiavalicarb-isopropyl were discussed. One of the oversights is that oestrous cyclicity was not investigated in any study. Oestrous cyclicity was investigated in the parent animals and offspring in a two-generation rat study where no effects were noted. It was also stated that in rats effects were observed in the ovaries, which together with increased estradiol serum levels and increased incidence of uterine adenocarcinoma provided sufficient evidence to conclude that benthiavalicarb-isopropyl is an endocrine disruptor. However, there were no effects on ovaries in rats, no effects on estradiol serum levels in a specifically designed and valid *in-vivo* 8-weeks mechanistic rat study and no effect on the key indicator, *i.e.* E_2/P_4-ratio, for endocrine-mediated uterine tumorigenesis in the 2-year carcinogenicity and *in vivo* mechanistic studies. The point on inconsistencies and oversights is further discussed in Section 5.

Such oversights raise scepticism against EFSA's conclusion on the endocrine disrupting potential. Therefore an extensive expert review of the available toxicity data package was carried out, of which the outcomes are detailed in Section 3. The conclusion of this assessment is that benthiavalicarb-isopropyl causes no estrogen, androgen, steroidogenic and/or thyroid-mediated toxicity.

3 ASSESSMENT OF POTENTIAL ENDOCRINE DISRUPTING PROPERTIES

The potential endocrine disrupting properties of benthiavalicarb-isopropyl have been assessed in accordance with the recommendation in the joined ECHA/EFSA *Guidance for the identification of endocrine disruptors in the context of Regulation (EU) No 528/2012 and (EC) No 1107/2009* (June 2018), which is subsequently referred to as the ED GD.

3.1 Step 1: Extraction and reporting of the available information

An overview of all the studies performed on benthiavalicarb-isopropyl with information that is relevant for the assessment of endocrine disruption is given in Annex 1 of this report.

3.2 Step 2: Lines of evidence for endocrine activity and adversity

All toxicological information on benthiavalicarb-isopropyl that is relevant for the evaluation of potential endocrine activity and adversity has been organized by modality in tabular format and include the assessment of (1) lines of evidence for estrogenic, androgenic and steroidogenesis endpoints (EAS modalities) including the endpoints that are sensitive to, but not diagnostic of EAS-mediated effects (Annex 2), (2) lines of evidence for thyroid endpoints (T modality), including the endpoints that are sensitive to but not diagnostic of thyroid effects (Annex 3) and (3) lines of evidence related to general toxicity (Annex 4).

3.3 Step 3: Discussion of the integrated and assessed lines of evidence relating to toxicity

To assess whether the endocrine related effects are present at dose levels at or beyond a dose of excessive toxicity, it is important to derive the maximum tolerated dose (MTD) for short-term and long-term repeated dose oral toxicity studies in the experimental animal species investigated, *i.e.* mouse, rat, and dog.

3.3.1 Definition and derivation of Maximum Tolerated Doses (MTDs)

Although there is no strict definition, the MTD was initially based on a weight gain decrement observed in the sub-chronic study, *i.e.* the highest dose that caused no more than a 10% weight gain decrement (ISGC 1986; OECD 2012) and without causing death (OECD 2002). However, due to more recent evaluations of many more bioassays, the OECD and other public organisms gave it a larger definition covering more effects than those given above and indicated MTD selection refinements on the basis of a broader range of biological information.

Hence, in the Environmental Health Perspectives (ISGC 1986) the MTD is indicated by “alterations in body and organ weight and clinically significant changes in hematologic, urinary and clinical chemistry measurements can be useful in conjunction with the usually more definitive toxic, pathologic or histopathologic endpoints.”

In OECD GD No.116 (2012), it is stated that when “the main objective of the study is to identify a cancer hazard, there is broad acceptance that the top dose should ideally provide some signs of toxicity such as slight depression of body weight gain (not more than 10%), without causing *e.g.*, tissue necrosis or metabolic saturation and without substantially altering normal life span due to effects other than tumours.”

In OECD TG 483 ([1]), the MTD is described as “the dose inducing slight toxic effects (for example, abnormal behaviour or reactions, minor body weight depression or hematopoietic system cytotoxicity) but not death or evidence of pain, suffering or distress necessitating humane euthanasia.” To be noted, OECD GD No. 19 (2000) explores the endpoint considered as humane endpoints for toxicological studies.

The US Interagency Staff Group on Carcinogens defined the MTD as "the highest dose currently recommended *[…]* which, when given for the duration of the chronic study, is just high enough to

([1]) OECD TG 483 (2015): Mammalian Spermatogonial Chromosomal Aberration Test

elicit signs of minimal toxicity without significantly altering the animal's normal lifespan due to effects other than carcinogenicity; *[…]* is determined in a subchronic study (usually 90 days duration) primarily on the basis of mortality, toxicity and pathology criteria; *[…]* should not produce morphologic evidence of toxicity of a severity that would interfere with the interpretation of the study; nor should it comprise so large a fraction of the animal's diet that the nutritional composition of the diet is altered, leading to nutritional imbalance.(ISGC 1986)"

From all of the above, it is clear that deriving proper MTDs goes beyond the historical definition of a 10% decrease in body weight gain or mortality and that expert judgement must be given as to evaluate whether the systemic and toxic effects observed are beyond what should be considered as "slight toxic effects" as there is no exhaustive list of these parameters.

The MTDs were evaluated according to the above definitions, for the (1) 13-weeks oral study in mice ([redacted], 1998a), (2) 2-year carcinogenicity study in mice ([redacted], 2001b), (3) 13-weeks oral toxicity study in rats ([redacted], 1998b), (4) 2-year carcinogenicity study in rats ([redacted], 2001a), (5) 13-weeks oral toxicity study in dogs ([redacted], 1999) and (6) 1-year oral toxicity study in dogs ([redacted], 2001).

13-weeks oral toxicity study in mice ([redacted], 1998a)

Body weight gain calculated over the entire treatment period of 13 weeks was statistically significantly decreased in males by 32% and 43% at 7,000 and 20,000 ppm respectively. From the histopathological examination of the tissues after treatment for 13 weeks statistically significant changes were observed in the liver and ovaries. In the liver anisonucleosis was observed at dose levels from 7,000 ppm in males, fatty change at 20,000 ppm in males, hepatocytic hypertrophy from 7,000 ppm in males and females, multinucleated giant cells at 20,000 ppm in males, necrosis from 7,000 ppm in males and females and bile duct proliferation at 20,000 ppm in males and females. In the ovaries there was a decrease in *corpora lutea* at a dose level of 20,000 ppm in females. From these observations it is clear that benthiavalicarb-isopropyl apart from organs and tissues that are sensitive to endocrine changes, such as the ovaries, produces significant adverse effects on body weight gain in the male mice and in the liver at dose levels of 7,000 and 20,000 ppm in male and female mice. Therefore the MTD for a treatment of mice for 13 weeks should be set at 7,000 ppm.

2-year carcinogenicity study in mice ([redacted], 2001b)

A statistically significant increase in mortality was seen in males at a dose level 5,000 ppm. Body weight gain calculated over the treatment period of 104 weeks was statistically significantly decreased in males by 26% and 30% at 2,500 and 5,000 ppm respectively. From the histopathological examination of the tissues after 104 weeks of treatment statistically significant changes were observed in the bone marrow, stomach, liver, thyroid, ovaries, uterus and adrenals. In the bone marrow there was an increase in megacaryocytes at 5,000 ppm in the males. Forestomach ulcers, lymphocytic infiltration and squamous cell hyperplasia were observed at 2,500 and 5,000 ppm in the males. Hepatocellular adenoma were observed at 2,500 and 5,000 ppm in males and females, hepatocellular carcinoma and hepatoblastoma at 2,500 and 5,000 ppm in males, hepatocytic hypertrophy at 2,500 and 5,000 ppm in males and females, intermediate fatty change and foci of cellular alteration at 2,500 and 5,000 ppm in males and females, anisonucleosis at 2,500 and 5,000 ppm in males and at 5,000 ppm in females, necrosis at 2,500 and 5,000 ppm in males and at 5,000 ppm in females, single cell necrosis at 2,500 and 5,000 ppm in males and at 5,000 ppm in females, lymphocytic infiltration, multinucleated hepatocytes, accumulation of macrophages, bile duct proliferation, extramedullary hematopoiesis and fibrosis at 2,500 ppm in males. Follicular cell hyperplasia was observed at 2,500 and 5,000 ppm in males and females, follicular cell adenoma at 5,000 ppm in males. Ovarian atrophy was reported at dose levels at 2,500 and 5,000 ppm in females. Uterine angiectasis was observed at 5,000 ppm in females. In the adrenals, cortical hypertrophy was observed at 2,500 and 5,000 ppm in males and females. From these observations it is evident that benthiavalicarb-isopropyl apart from organs/tissues sensitive to endocrine changes, such as the ovaries, uterus, adrenals and thyroid, produced significant adverse effects on body weight gain in the males, in the stomach in the males

and in the liver at 2,500 ppm and 5,000 ppm in males and females. Therefore the MTD for a treatment of 104 weeks in the mouse should be set at 2,500 ppm.

13-weeks oral toxicity study in rats including a 4-week recovery period ([redacted]e, 1998b)

There was no effect on mortality and body weight. From the histopathological examination of the tissues after treatment for 13 weeks statistically significant changes were observed in the liver and kidneys. Hepatocytic hypertrophy was observed at 20,000 ppm in males and females. Renal mineralisation was observed at 20,000 ppm in females. At the high dose of 20,000 ppm only relatively mild effects were observed in the liver and in the kidney. Therefore the MTD for a treatment of 13 weeks in the rat can be considered to be higher than 20,000 mg/kg body weight.

2-year carcinogenicity study in rats ([redacted], 2001a)

No meaningful effect on body weight and body weight gain was noted. From the histopathological examination of the tissues after treatment for 104 weeks statistically significant changes were observed in the pancreas, liver and kidneys. Atrophy of the exocrine pancreas was observed at 10,000 ppm in males and females. Hepatocellular adenoma was observed at 10,000 ppm in males, fatty change at 5,000 and 10,000 ppm in females, *spongiosis hepatis* at 5,000 and 10,000 ppm in males, hepatocytic hypertrophy at 10,000 ppm in males and at 5,000 and 10,000 ppm in females. Glomerulosclerosis of the kidneys was observed at 5,000 and 10,000 ppm in females, calculus at 5,000 and 10,000 ppm in males and females, chronic nephropathy at 5,000 and 10,000 ppm in males, brown pigment deposition at 5,000 and 10,000 ppm in females, dilated tubules at 5,000 ppm and 10,000 ppm in males, hyaline droplets at 5,000 and 10,000 ppm in males and at 10,000 ppm in females, lymphocytic infiltration at 5,000 and 10,000 ppm in females, fibrosis and transitional cell hyperplasia at 10,000 ppm in males. Uterine adenocarcinoma was observed in females at 5,000 and 10,000 ppm. From these observations it is evident that benthiavalicarb-isopropyl apart from organs/tissues sensitive to endocrine changes, such as the uterus, produced significant adverse effects in the liver and the kidneys at 5,000 and 10,000 ppm. Therefore the MTD for a treatment of 104 weeks in the rats should be set at 5,000 ppm.

13-weeks oral toxicity study in dogs ([redacted], 1999)

No effect was noted on mortality and body weight. From the histopathological examination of the tissues after 13 weeks of treatment statistically significant changes were observed in the liver. These included deposition of pigment and hepatocytic hypertrophy at 1,000 mg/kg bw in males and females. At the maximum dose of 1,000 mg/kg bw only relatively mild effects were observed in the liver. Therefore the MTD for a treatment of 13 weeks in the dog can be considered to be greater than 1,000 mg/kg bw.

1-year oral toxicity study in dogs ([redacted], 2001)

No effect was noted on mortality and body weight. From the histopathological examination of the tissues after 52 weeks of treatment only a slight increase in the incidence of pituitary cysts was noted in females without a clear dose-response relationship. Therefore, the MTD for a treatment of 52 weeks in the dog can be considered to be greater than 400 mg/kg bw.

Conclusion

An overview of the MTDs in short- and long-term toxicology studies in the mouse, rat and dog is given in Table 3.3-1.

Table 3.3-1 Maximum Tolerated Doses in mouse, rat and dog toxicity studies

Species / Exposure Duration	13 weeks	52 weeks	104 weeks
mouse	7,000 ppm	-	2,500 ppm
rat	>20,000 ppm	-	5,000 ppm
dog	> 1,000 mg/kg bw	> 400 mg/kg bw	-

The ED Guidance states that "Where potentially endocrine-related adverse effects are only observed at excessive toxic dose/concentration (*i.e.* only observed above the MTD or MTC) they should not be considered indicative of endocrine disruption." Although the adverse effects observed at excessive toxicity must not be considered in the assessment of potential endocrine disrupting properties, these effects were taken into account in the assessment in order to dismiss any doubts.

3.3.2 Assessment of the endocrine sensitive apical endpoints identified in the lines of evidence

3.3.2.1 Estrogen, Androgen and Steroidogenesis (EAS) Modalities

From the lines of evidence for EAS-mediated adversity it can be concluded that benthiavalicarb-isopropyl does not produce effects in experimental animals on the following 'EATS-mediated' parameters: oestrous cyclicity, uterus weight, gravid uterus weight, uterus histopathology except angiectasis, which is a swelling of the blood vessels and not related to endocrine disruption ([2]), uterine adenocarcinoma, ovary weight except in mice, ovary histopathology except in mice, vaginal opening, vaginal histopathology, mammary gland histopathology, epididymis weight, epididymis histopathology, preputial separation, testicular weight except in aged rats, testicular histopathology, seminal vesicles weight and histopathology, prostate weight, prostate histopathology, sperm activity, sperm motility, sperm count except in rats and sperm morphology.

No effects have been found on following parameters that are 'sensitive to, but not diagnostic of EAS': copulation index, fertility index, gestation period, gestation index, number of implantation sites, pre-implantation loss, post-implantation loss, number of live foetuses, number of dead foetuses, number of new-borns, number of live pups, number of dead pups, sex ratio, delivery index, live birth index, viability index at day 4, weaning index, eruption of lower incisors, pituitary weight and pituitary histopathology except in mice and dogs.

The effects on EAS parameters observed in experimental animals that are the exception of the generally negative endocrine disruption profile of benthiavalicarb-isopropyl are assessed for their potential endocrine disruption here below.

Increase in estradiol serum levels in the long-term rat study ([redacted], 2001a)

Because of the increased incidence in uterine adenocarcinomas at 5,000 and 10,000 ppm, estradiol and progesterone were measured in cryopreserved serum from 10 females from the control group and 10 females from the 5,000 ppm and 10,000 ppm dose groups each sacrificed at 26, 78 and 104 weeks of dosing. The time points in the oestrus cycle during sampling were not specified. The determination of both hormones was made by ACS 180 PLUS (Bayer Medical Ltd.) using the CLIA method, which was validated by Mukai (1999). A copy of the original certificate in Japanese language is included in Annex 5 and the English translation thereof in Annex 6 of this document.

Estradiol (E_2) serum levels were statistically significantly increased in week 26 (27% at 5,000 ppm and 38% at 10,000 ppm) and in week 78 (70% at 5,000 ppm and 40% at 10,000 ppm) without a consistent dose effect relationship (Table 3.3-2). No increase in estradiol serum levels was noted in week 104. There was no statistically significant change in progesterone (P_4) serum levels and the E_2/P_4-ratio. It is of note that the E_2/P_4 ratio is the most important indicator for uterine adenocarcinoma (Nagaoka *et al*. 1990; Yoshida *et al*. 2012; Klaunig *et al*. 2016; Van Cott *et al*,. 2018). There were slight and inconsistent changes in estradiol serum levels measured at three time points during the 104-week treatment period.

The effect of benthiavalicarb-isopropyl on serum E_2 levels was not confirmed in a repeated dose toxicology study ([redacted], 2002c) with measurements at (pre-dosing) and at weeks 2, 4, 6 and 8 of dosing during the di-oestrous phase (Table 3.3-3). It can therefore be concluded that the changes in

([2]) In Murata 2001b: Angiecatsis is a dilatation of the blood vessels. Dilatation of the uterus lumen and gland were also investigated in the study under the histopathology names "dilatation, lumen" and "dilatation, gland". No substance related effect was observed at any of the tested doses and sampling time.

the E_2 levels noted in week 26 and week 78 of the 2-year carcinogenicity study are of limited or of no pathological significance, in particular, in the absence of any ovarian and vaginal pathology.

Table 3.3-2 Hormone levels in 10 female rats at 26, 78 and 104 week (mean ± standard deviation) from the 2-year carcinogenicity study ([redacted], 2001a)

Hormone	Dose level [ppm]	Sampling time [weeks]		
		26	78	104
estradiol [pg/mL]	0	49.0 ± 12.7	35.2 ± 15.5	19.7 ± 8.0 [a]
	5000	**62.3 ± 14.3 ***	**60.1 ± 17.3***	24.3 ± 10.5 [b]
	10000	**67.7 ± 14.5 ****	**49.2 ± 11.7 ****	25.9 ± 11.8
progesterone [ng/mL]	0	26.5 ± 15.6	44.7 ± 33.4	77.0 ± 49.9
	5000	25.6 ± 16.3	37.2 ± 36.0	36.5 ± 24.3
	10000	33.8 ± 21.4	47.6 ± 41.3	43.4 ± 48.1
E_2/P_4 [$\times 10^{-3}$]	0	2.52 ±1.49	1.09 ± 0.78[n]	0.7 3± 1.35
	5000	3.42 ± 1.85	3.49 ± 3.29	1.24 ± 0.93
	10000	2.75 ± 1.45	1.79 ± 1.26	1.08 ± 0.90

* $p \leq 0.05$; ** $p \leq 0.01$; [a]: n=8 (two animals below 10 pg/mL were excluded); [b]: n=9 (one animal below 10 pg/mL was excluded); [n]: non parametric analysis

Table 3.3-3 Hormone levels in 10 female rats at 2, 4, 6 and 8 weeks (mean ± standard deviation) obtained during the di-oestrous phase from an 8-weeks *in vivo* mechanistic study ([redacted], 2002c)

Hormone	Dose level [ppm]	Sampling time [weeks]			
		2	4	6	8
estradiol [pg/mL]	0	40.1 ± 21.0	25.0 ± 16.7	21.0 ± 16.1	29.9 ± 22.7 [n]
	200	40.5 ± 30.9	24.1 ± 15.7	22.0 ± 18.7	30.5 ± 9.7
	10000	39.2 ± 20.4	32.4 ± 12.3	26.6 ± 15.6	26.2 ± 13.6
progesterone [ng/mL]	0	6.6 ± 3.2 [n]	6.3 ± 4.4 [n]	4.6 ± 1.5	18.0 ± 13.0
	200	7.3 ± 6.3	4.2 ± 0.7	5.0 ± 1.2	21.8 ± 12.8
	10000	5.1 ± 1.1	5.3 ± 2.0	5.0 ± 0.9	26.5 ± 15.7
E_2/P_4 [$\times 10^{-3}$]	0	7.53 ± 4.45	5.49 ± 4.42	4.83 ± 3.96	2.32 ± 2.40
	200	8.04 ± 6.07	5.62 ± 2.99	4.65 ± 3.99	1.98 ± 1.43
	10000	8.22 ± 4.86	6.99 ± 3.62	5.77 ± 4.54	1.57 ± 1.91
Luteinizing Hormone [ng/mL]	0	5.4 ± 1.2	10.4 ± 3.3	4.7 ± 1.5	9.2 ± 2.5
	200	5.5 ± 0.9	8.8 ± 2.2	5.6 ± 2.0	8.4 ± 2.5
	10000	5.2 ± 1.4	11.0 ± 2.5	5.5 ± 0.9	10.4 ± 1.6

*$p \leq 0.05$; ** $p \leq 0.01$; [n]: non parametric analysis

Increase in the incidence of adenocarcinoma of the uterus of the rat ([redacted], 2001a)

The incidences of uterine adenocarcinoma observed in F344:DuCrj rats treated for 104 weeks with benthiavalicarb-isopropyl are shown in Table 3.3-4. In the female F344 rat a statistically significant increase in the incidence of uterus adenocarcinoma was seen at the two highest dose levels (22% at 5,000 ppm and 20% at 10,000 ppm) with no dose-response relationship at these dose levels. These incidences are higher than the historical control range of the test laboratory in the period from 1996 to 2005 (0.0-8.0%). The historical control database of the test laboratory for that time period covers 15 long-term carcinogenicity studies and 750 animals from the control groups ([redacted], 2017). In this assay no pre-neoplastic lesions of uterus adenocarcinoma were found in week 78 and week 104. In order to understand any underlying mode of action of the production of uterine adenocarcinoma in rats two *in vivo* mechanistic studies were performed.

Table 3.3-4 Incidences of uterine adenocarcinoma (%) in the F344 rat at 104 weeks

	Concentration of benthiavalicarb-isopropyl in the diet (ppm)

	0	50	200	5,000	10,000
females	6	6	8	**22***	**20***

* $p \leq 0.05$

Known hormonal and non-hormonal mechanisms for uterine adenocarcinoma in rats are (1) genotoxicity, (2) direct estrogenicity, (3) interference with the hormone balance such as by liver enzyme induction and (4) dopamine agonism (Klaunig *et al*. 2018). Benthiavalicarb-isopropyl is not mutagenic. Direct estrogenicity and alteration of estrogen biotransformation are discussed in the following paragraphs and are concluded to be not applicable to benthiavalicarb-isopropyl. Indirect modulation of estrogen concentrations by benthiavalicarb-isopropyl acting as a dopamine receptor agonist is also unlikely considering that there is no increase of the E_2/P_4-ratio.

In an 8 week *in vivo* mechanistic study ([redacted], 2002c) ten female F344 rats were treated with benthiavalicarb-isopropyl via the diet at concentrations of 0, 200, and 10,000 ppm for a period of 8 weeks. Observations were made for (a) body weight, (b) food consumption, (c) aromatase activity in liver, uterus and ovaries, (d) di-oestrous phase concentrations of estradiol (E_2), progesterone (P_4) and luteinizing hormone (LH) in serum at pre-dosing and at weeks 2, 4, 6 and 8 of dosing, (e) gross necropsy (f) and organ weight. Determination of estradiol and progesterone was also made by ACS 180 PLUS (Bayer Medical Ltd.) using CLIA method (validation in Mukai 1999a and Mukai 1999b) and luteinizing hormone was measured through Rat LH ETA system (Amersham) (ELISA kits). Both were systems commercially available. The method for aromatase activity measurement is described in [redacted](2002c). There were no significant differences in mean body weight between the dose groups and the controls throughout the dosing period. Aromatase activity was statistically significantly increased in the liver at 10,000 ppm but not in the ovaries and the uterus when compared to the control group (Table 3.3-5).

Table 3.3-5 Aromatase activity in ovary, uterus and liver in 10 female rats at 8 weeks of dosing (mean ± standard deviation)

Dose level [ppm]	Aromatase activity [pmol/mg/h] at 8 weeks		
	Ovary	Uterus	Liver
0	0.89 ± 0.16	19.4 ± 7.8	3.08 ± 0.49
200	0.88 ± 0.14	19.9 ± 8.6	2.99 ± 0.85
10000	0.80 ± 0.11	22.4 ± 15.9	4.20 ± 0.97 **

** $p \leq 0.01$

Aromatase plays an important role in the biosynthesis of estradiol in the ovary where testosterone produced by the theca cells of the pre-antral and antral follicles is converted to estradiol in the granulosa cells. There were no significant differences in serum concentrations of estradiol, progesterone or LH between the dosed groups and the controls. Absolute and relative liver weight was statistically significantly increased in the 10,000 ppm dose group when compared to the controls, which is in accordance with the liver enzyme induction properties of benthiavalicarb-isopropyl. There were no changes in absolute and relative weights of the ovaries and the uterus in the 10,000 ppm dose group. Necropsy revealed enlarged livers in the dosed groups. The lack of any change in serum estradiol, progesterone or LH levels at several intervals during treatment suggests that benthiavalicarb-isopropyl does not interfere with the biosynthesis or metabolism of these hormones in rats treated for up to 8 weeks.

In an *in vivo* uterotrophic bioassay ([redacted], 2015) benthiavalicarb-isopropyl was administered to ovariectomised rats by gavage at doses of 10, 100 and 1,000 mg/kg bw/day for a period of 14 days. Benthiavalicarb-isopropyl had no effects on uterine weight, vaginal weight and there were no histopathological lesions or cell proliferation in the uterine and vaginal tissues. These findings confirm that benthiavalicarb-isopropyl does not possess estrogenic properties.

According to Yoshida *et al*. (2015), possible hormone-related Modes of Actions for the development of endometrial cancer in ageing rats are (1) enhanced exposure to estradiol or exposure to estrogenic compounds, (2) increase of the E_2/P_4-ratio and (3) a modulation of estrogen metabolism through

induction of CYP1A1, CYP1A2 and CYP1B1 enzymes in the liver with the production of 2-hydroxyestradiol (2-OHE_2) and 4-hydroxyestradiol (4-OHE_2). These estradiol metabolites are suspected to play a role in carcinogenesis as a result of the oxidisation to quinone derivatives, *i.e*. 2,3-OHE_2-*o*-quinone and 3,4-OHE_2-*o*-quinone. Quinones have the potential to function at both the initiation and the promotion stage of the tumour formation process in rodents.

The study of [redacted] (2002c) demonstrated that benthiavalicarb-isopropyl does not increase serum estradiol levels in rats exposed to a diet containing up to 10,000 ppm for up to 8 weeks. Estrogenic activity can be excluded considering that benthiavalicarb-isopropyl has been tested in the uterotrophic assay in ovariectomised rats at dose levels up to 1,000 mg/kg bw/day for a period of 14 days without showing any effect ([redacted], 2015). Also no alteration in oestrous cyclicity has been observed in the two-generation reproduction toxicity study in rats exposed to dietary concentrations up to 10,000 ppm ([redacted], 1999) and no histopathological changes were evident in the ovaries and in the vaginal epithelium of rats in the long-term carcinogenicity study ([redacted], 2001a).

The E_2/P_4-ratio was measured after 8 weeks of treatment at dietary concentrations up to 10,000 ppm ([redacted], 2002c) and after 26, 78 and 104 weeks of treatment up to 5,000 ppm ([redacted], 2001a). In both studies no changes were observed.

The possible modulation of oestrogen metabolism through activation of the AhR receptor and subsequent induction of CYP1A1, CYP1A2, and CYP1B1 enzymes in the liver was addressed in an *in vivo* study on the induction of drug-metabolizing enzymes and cell proliferation in hepatocytes of rats (Murata, 2001c). F344 rats were dosed daily with benthiavalicarb-isopropyl up to 1,000 mg/kg bw for 7 days. Five animals/sex/dose were subjected to the measurement of CYP1A1(1A2) enzymes and a slight but statistically significant 1.6-fold increase in CYP1A1(1A2) enzymes was measured. This increase is very small and not typical for AhR activators. For example, the classical AhR ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin is reported to induce the CYP1A1 enzyme more than 1000-fold in cultures of human hepatocytes (Budinsky *et al*., 2010). Furthermore, there is no evidence for oxidative damage to DNA in the F344 rats ([redacted], 2001a). Oxidative DNA damage may be the consequence of oxidative stress produced by 2,3-OHE_2-*o*-quinone and 3,4-OHE*2*-*o*-quinone. If, benthiavalicarb-isopropyl would induce estradiol oxidative metabolism, this would have an effect on estradiol serum levels but this is not the case ([redacted], 2002c).

None of the three possible MoAs for endometrial cancer proposed by Yoshida *et al*., 2015 operate in the rat. Adenocarcinoma development in the uterus of Donryu rats, a rat strain which is particularly sensitive to endometrial cancer, is associated with increased serum E_2/P_4-ratio (Nagaoka *et al*., 1990). Following a comparative investigation of strain differences, Nagaoka *et al*. (1990) confirmed that "irregular estrous cycles began earlier with higher incidence in Donryu rats than in F344 rats, a low-incidence strain. Histological findings of the ovary and vaginal epithelium also suggested relatively increased estrogen levels in Donryu rats compared to F344 rats. Estimated plasma values of gonad steroids showed that the E_2/P_4-ratio in Donryu rats at 12 months of age was about five times that in F344 rats. These results therefore indicate that hormone imbalance, particularly an increased E_2/P_4-ratio, may play an important role in the spontaneous occurrence of endometrial adenocarcinoma in Donryu rats". Although in the carcinogenicity assay with the F344 rat there was an increase in the incidence of uterine adenocarcinoma at 5,000 and 10,000 ppm ([redacted], 2001a), no meaningful and consistent increase in estradiol, progesterone and E_2/P_4-ratio was evident. Moreover, there were no histopathological changes in the ovaries and the vaginal epithelium. It can therefore be concluded that no endocrine-related MoA has been identified for the uterine adenocarcinoma in the rat.

Effects on ovary weight and histopathology in the mouse ([redacted], 2001b; [redacted], 1998a)

In the 2-year mouse carcinogenicity study [redacted], 2001b) a statistically significant decrease in absolute ovary weight was observed in week 104 (11% at 2,500 ppm and 33% at 5,000 ppm) but not in weeks 52 and 78. There was no effect on relative ovary weight, which can be attributed to a decrease in body weight at the time of sacrifice. Atrophy of the ovaries was noted at 2,500 ppm and 5,000 ppm but there was no substance-related decrease in the number of *corpora lutea*. Since no effect on absolute ovary weight was observed in weeks 52 and 78 it is highly likely that the loss in

ovary weight and ovarian atrophy in week 104 is related to the exacerbation of the senescence process of the reproductive organs in female mice by benthiavalicarb-isopropyl. It needs to be emphasized that these effects have been observed at dose levels with excessive toxicity and that no such effects have been observed in the two-generation reproductive toxicity study and in the rat carcinogenicity study.

In a mouse sub-chronic oral toxicity study ([redacted], 1998a) a statistically significant decrease in absolute (29%) and relative (23%) ovary weight was noted in week 13 at 20,000 ppm. At the same dose level all females had a reduced number of *corpora lutea*. The reduction in the number of *corpora lutea* in the ovaries of female mice treated at 20,000 ppm for 13 weeks are of limited pathological significance because the statistically significant increase of incidence was noted at the highest tested dose level of 20,000 ppm showing excessive toxicity and no substance-related reduction of *corpora lutea* was observed in the reproductive toxicity and two-year carcinogenicity studies.

Effects on prostate weight and histopathology in the dog ([redacted], 1999)

Benthiavalicarb-isopropyl was administered to groups of four beagle dogs per sex in gelatine capsules at doses of 0, 40, 200 and 1000 mg/kg/bw for a period of 13 weeks. A non-statistically significant decrease in absolute and relative prostate weight was recorded. The prostates of dogs of 5 months of age are still immature and the weight is highly variable and therefore no clear dose/effect relationship could be established. Histopathology did not show any substance-related effect. Since no such effect was observed in dogs in the 4-weeks ([redacted], 1998) and 52-weeks ([redacted]a, 2001) repeated dose toxicology studies, it can be concluded that the effect on prostate weight observed in the 13-weeks dog toxicity study has no pathological significance.

Effect on testes weight in the rat ([redacted], 2001a)

In the two-year carcinogenicity study in rats a very slight but statistically significant increase in absolute testes weight without dose/effect relationship was noted after 26 weeks of treatment at 5,000 (5%) and 10,000 ppm (2%). No change in absolute testicular weight was evident after 52, 78 and 104 weeks of treatment and no substance-related effect was noted on testes and prostate histopathology at dose levels up to 10,000 ppm. In the two-generation reproduction toxicity study no substance-related effect on absolute and relative weight and histopathology of the seminal vesicles was reported in the F_0 and F_1 generations exposed to dietary concentrations up to 10,000 ppm. In the same study no substance-related effect on absolute and relative weight and histopathology of the prostate was noted the in F_0 and F_1 generations at any dietary concentration for up to 10,000 ppm ([redacted], 1999).

Since no androgenic effects have been observed in the male reproductive tissues in the two-year carcinogenicity and in the two-generation reproductive toxicity studies in rats, it can be concluded that the increased testes weight in the rat after 26 weeks of treatment is a spurious finding, which is not related to an endocrine activity.

Effects on sperm count in the rat ([redacted], 1999)

In the two-generation reproduction toxicity study in the rat a statistically significant decrease of the number of sperm in the *cauda epididymis* was reported at 100 ppm (38%) and 10,000 ppm (22%). No effect was however noted at 1,000 ppm in the F_1 generation and therefore there is no dose/effect relationship. In the F_0 generation a statistically significant increase (27%) in sperm count was recorded at 1,000 ppm whereas there was no change at 100 and 10,000 ppm. Since no consistent effect on sperm count could be established it can be concluded that these effects are spurious and of no value to endocrine disruption assessment.

Effects on pituitary histopathology ([redacted], 2001b; [redacted], 2001)

A slight but statistically significant increase in the incidence of male mice with pituitary cysts was observed after 104 weeks of treatment at 2,500 and 5,000 ppm. When benthiavalicarb-isopropyl was administered to dogs at 400 mg/kg bw for 52 weeks a tendency of an increased incidence of pituitary cysts was noted in males and females. In both studies no clear dose/response relationship was evident.

This slight increase in the incidence in pituitary cysts in male mice was however not observed in female mice and in male and female rats in the two-year carcinogenicity studies. Although the pituitary is an endocrine organ and regarded as a parameter "sensitive to, but not diagnostic of EATS", the presence of cysts in a limited number of male mice, i.e. 4out of 35 animals at 2,500 ppm and 4 out of 28 animals at 5,000 ppm, should not be regarded as having a role in endocrine disruption.

3.3.2.2 **Thyroid (T) modality**

A statistically significant increase in the incidence of thyroid follicular cell tumours was only found in male mice when treated for 104 weeks with benthiavalicarb-isopropyl in the diet ([redacted], 2001a; [redacted], 2001b). The incidences of thyroid follicular cell adenoma and carcinoma observed in F344 rats and B6C3F1 mice treated for 104 weeks with benthiavalicarb-isopropyl in the diet are shown in Table 3.3-6 ([redacted], 2001a) and Table 3.3-7 ([redacted], 2001b).

Table 3.3-6 Incidences of thyroid follicular cell tumours (%) in F344 rats at 104 weeks

	follicular cell tumours	Concentration of benthiavalicarb-isopropyl in the diet (ppm)				
		0	50	200	5,000	10,000
males	adenoma	0	2	0	0	0
	carcinoma	0	4	0	0	4
females	adenoma	0	0	0	0	2
	carcinoma	0	0	2	0	0

Although no thyroid follicular cell tumours were observed in male and female rats, 40% of the males and 50% of the females at 5,000 ppm and 90% of the males and 80% of the females at 10,000 ppm were demonstrated to have thyroid follicular hyperplasia.

Table 3.3-7 Incidences of thyroid follicular cell tumours (%) in the B6C3F1 mouse at 104 weeks

	follicular cell tumour	Concentration of benthiavalicarb-isopropyl in the diet (ppm)				
		0	20	100	2,500	5,000
males	adenoma	0	2	0	8	**18****
	carcinoma	0	2	0	0	0
females	adenoma	0	0	2	4	4
	carcinoma	0	0	0	0	0

** $p \leq 0.01$

A statistically significant increase in the incidence of thyroid follicular cell adenoma of 18% was observed at 5,000 ppm in male mice. At the same dose level almost 100% of the males showed follicular cell hyperplasia, which is a non-neoplastic precursor lesion of thyroid tumours. Although no statistically significant increase in the incidence of follicular cell tumours was noted in female mice, there was evidence of thyroid follicular cell hyperplasia at the two highest dose levels (50% at 2,500 ppm and 60% at 5,000 ppm).

In general, liver enzyme inducers producing hepatocellular tumours in rodents also produce follicular epithelial cell hyperplasia and ultimately follicular cell tumours in the thyroid. The induction of the UDP-GT enzyme that conjugates the thyroid hormones T3 and T4 with glucuronic acid causes an enhanced excretion of these hormones in the bile. The ensuing decrease in plasma T3 and T4 levels triggers a feedback mechanism whereby the production of thyroid stimulating hormone (TSH) by the anterior pituitary is induced (hypothalamo-pituitary-thyroid axis) to restore the levels of T3 and T4. Continuous stimulation of the thyroid by TSH leads to thyroid follicular hyperplasia and ultimately to thyroid follicular adenoma and carcinoma. Since rodents do not have thyroid hormone binding globulin, rodents are very sensitive to changes in plasma thyroid hormone levels. This mechanism, of which the induction of UDP-GT is based on the activation of the constitutive androstane nuclear receptor (CAR) is recognized to be of no relevance to man (Zoeller *et al.*, 2007). To address the relevance to man of the thyroid tumours mechanistic studies have been performed in the rat and in the mouse. This mode of action was substantiated in five mechanistic studies.

In a first *in vivo* mechanistic study ([redacted], 2002a) F344 rats were dosed daily in the diet at 0, 200 and 10,000 ppm for 14 days. UDP-GT activity toward T4 and serum concentrations of TSH, T3 (total and free) and T4 (total and free) were measured after 7 and 14 days of exposure. A slight but statistically significant increase of 16% in the activity of UDP-GT was found at 10,000 ppm and a statistically significant decrease of 15% and 18% in serum levels of T4 was noted after 7 and 14 days of treatment, respectively. No increase in TSH level and thyroid size were observed.

In a second *in vivo* mechanistic study ([redacted], 2002b) B6C3F1 mice were dosed daily in the diet at 0, 100 and 5,000 ppm for 7 and 14 days. UDP-GT activity toward T4 and serum concentrations of TSH, T3 (total and free) and T4 (total and free) were measured after 7 and 14 days of exposure. A statistically significant increase of 65% in the activity of UDP-GT was found at 5,000 ppm and a statistically significant decrease of 29% and 27% in serum levels of T4 was noted after 7 and 14 days of treatment, respectively. No increase in serum TSH level and thyroid size were observed. In a follow-up study ([redacted]i, 2003) B6C3F1 mice were divided into groups of 36 animals and exposed to benthiavalicarb-isopropyl in the diet at 0, 100 and 5,000 ppm for 2, 4, 8 and 16 weeks of treatment. Blood was collected after each exposure period for the determination of TSH in serum. Only at the end of the treatment period at week 16 a slight but statistically significant increase of 14% in TSH levels was noted at 5,000 ppm.

An *in vivo* mechanistic study ([redacted], 2018a) was conducted consisting of two arms, one with a treatment duration of 7 days and one with a treatment duration of 28 days, and two categories of parameters to be measured, i.e. one relating to Car/Pxr activation and one relating to thyroid hypertrophy and hyperplasia. In both arms of the study, groups of 15 wild type (WT) male C57Bl/6 mice were dosed with benthiavalicarb-isopropyl in the diet at 0, 500 ppm and 5,000 ppm or with diet containing 500 ppm phenobarbital (PB). Animals in both arms of the study were implanted with BrdU-containing osmotic pumps 7 days before termination to facilitate evaluation of cell proliferation. During the in-life phase of the study, body weight changes, food consumption and clinical observations were made. Upon study termination, the parameters that were evaluated included hepatic microsomal T4 glucuronidation activity, total T4 and T3 levels in plasma, gene expression of thyroid stimulating hormone beta (*Tshb*) and thyrotropin releasing hormone receptor (*Trhr*) in the pituitary, histopathology of the thyroid and the pituitary, plasma levels of thyroid stimulating hormone (TSH) and thyroid follicular cell proliferation. Car activation resulted in the induction of thyroid hormone glucuronidation and sulfation pathways in mice leading to the enhanced excretion of T4/T3-glucuronide in the bile and reduced systemic levels of T4 and T3. Benthiavalicarb-isopropyl induced hepatic microsomal T4 glucuronidation activity significantly in a dose-dependent manner in mice. Average increases were 0.3- and 1.3-fold relative to control levels at dietary concentrations of 500 and 5,000 ppm, respectively. PB at a dietary concentration of 500 ppm elicited an average increase of 0.7-fold. Exposure to benthiavalicarb-isopropyl in the diet at 5,000 ppm led to dose-dependent elevations in total T4 plasma levels, *i.e.* 0.9- and 0.7-fold increase after 7 and 28 days, respectively. A similar result was obtained with PB where total T4 plasma levels were increased 0.4-fold and 0.3-fold at 7 and 28 days, respectively. The reason for these unanticipated findings remains unclear but may relate to feedback control mechanisms over-compensating for reduced systemic T4 levels occurring at earlier time-points. Plasma levels of total T3 remained unchanged at any of the evaluated time points in response to either benthiavalicarb-isopropyl or PB. The histopathological examination of thyroid tissue after 7 or 28 days of treatment showed an unexpected change, which could be an accumulation of thyroglobulin within the follicular cells. Due to the obscuring effect of this change there was no convincing evidence of any treatment-related histopathological effect. The histological examination of the pituitary gland revealed an increased incidence of hypertrophic basophilic cells, which is consistent with an increased secretion of peptides including luteinising hormone (LH), follicle stimulating hormone (FSH), corticotrophs and thyrotrophs. Pituitary expression of both *Tshb* and *Trhr* genes was induced by benthiavalicarb-isopropyl in a dose-dependent manner and appeared to be a delayed response as it was only observed after 28 days of treatment at 5,000 ppm with a 3.8- and 1.3-fold increase of *Tshb* and *Trhr*, respectively. PB also induced the transcription of these genes only after 28 days of treatment with a 1.6- and 0.8-fold increase for *Tshb* and *Trhr*, respectively. There was a tendency of increased TSH plasma levels at 7 days but this could not be demonstrated at 28 days despite the increased *Tshb* expression in the pituitary. The mouse-to-mouse variation in TSH levels

was greater than anticipated and this experiment may not have been powerful enough to detect modest increases in the levels of this peptide. Only thyroid follicular cell proliferation frequency was found to be statistically significantly increased by benthiavalicarb-isopropyl after 28 days of treatment with a 31 and 51% increase at 500 and 5,000 ppm, respectively. Cell proliferation frequency was statistically significantly increased by PB with 35 and 54% after 7 and 28 days of treatment at 500 ppm, respectively.

To exclude other MoAs that could give rise to enhanced stimulation of the thyroid leading to hypertrophy, hyperplasia and ultimately tumours an *in vitro* assay ([redacted], 2018e) was carried out to investigate the inhibitory potential inhibitory effect of benthiavalicarb-isopropyl on thyroid peroxidase (TPO). The method used is based on the oxidation of guaiacol to a coloured substance by TPO present in female Yorkshire pig thyroid microsomes. The use of pig thyroid microsomes in this assay is justified since TPO is well-conserved across species, although there can be quantitative differences in inhibition constant. Microsomes were pre-incubated with guaiacol in assay buffer at 37°C in a 96-well plate and the reaction was initiated by addition of H_2O_2. The rate of oxidation of guaiacol was determined spectrophotometrically at 450 nm. The OD_{450} was followed over time and TPO activity expressed in units of ΔOD_{450}/min/mg protein. To measure the inhibitory potential, benthiavalicarb-isopropyl and the reference substance 6-propyl-2-thiouracil (PTU) were added to the assay system at 9 concentrations ranging from 0.01 to 100 µM. PTU was confirmed to be a potent inhibitor of TPO activity in this system with an estimated IC_{50} value of 12.2 µM. Benthiavalicarb-isopropyl did not inhibit porcine TPO at any appreciable extent in this *in vitro* test system at any of the concentrations tested. Inhibition of TPO can therefore be excluded as a MoA for thyroid hypertrophy, thyroid hyperplasia and thyroid tumour formation in the mouse.

Key events of thyroid tumour formation

The postulated MoA of benthiavalicarb-isopropyl for the induction of thyroid follicular cell tumours in the mouse involves activation of Car/Pxr (1st key event) leading to the induction of T4-UDP-GT in the liver (2nd key event). Enhanced conjugation of T4 and T3 with glucuronic acid increases the excretion of thyroid hormones via the bile with the reduction of T4 and T3 plasma levels as a consequence. Since rodents do not have thyroid hormone binding globulin, the change in plasma levels is instant and is immediately detected by the hypothalamus activating the hypothalamic-pituitary-thyroid (HPT) axis to restore thyroid hormone homeostasis. The hypothalamus produces thyrotropin releasing hormone (TRH) which binds to the TRH receptor in the pituitary increasing the production of thyroid stimulating hormone (TSH) by the basophilic cells in the anterior pituitary (3rd key event). TSH activates the thyroid to produce more thyroid hormone. Continuous stimulation of the thyroid by TSH leads to thyroid follicular cell hypertrophy and follicular cell proliferation (4th key event) which ultimately gives rise to the formation of thyroid tumours (5th key event) (Zoeller *et al*., 2007).

Car/Pxr activation (**KE1**) was demonstrated *in vivo* and *in vitro*. In the dietary study in male C57Bl/6 mice dosed up to 5,000 ppm benthiavalicarb-isopropyl in feed ([redacted], 2018a) *Cyp2b10* mRNA levels were statistically significantly induced in a dose-dependent manner with 450- and 1,900-fold increases at 500 and 5,000 ppm, respectively. Although increases in *Cyp3a11* mRNA levels were statistically significant and dose dependent, the expression increase marginally at 1.2- and 6.6-fold at 500 and 5,000 ppm, respectively. In an *in vitro* study using wild type mouse hepatocytes ([redacted], 2018b) no increase in *Cyp2b10* mRNA levels was seen whereas *Cyp3a11* mRNA levels increased by a 1.5-fold. The lack of a difference in induction of *Cyp2b10* transcription between control and treated wild type mouse hepatocytes may be due to a spontaneous onset of a progressive induction of the *Cyp2b10* gene in the 96-h *in vitro* assay and it might be that an increased induction of *Cyp2b10* expression in hepatocytes exposed to benthiavalicarb-isopropyl and PB would have been evident at shorter sampling times as an increase in the activity of the corresponding enzyme was observed. In an *in vitro* assay using hepatocytes from Car/Pxr-knockout mice no induction of *Cyp2b10* and *Cyp3a11* gene expression was observed ([redacted], 2018c), which indicates that benthiavalicarb-isopropyl operates through a Car-dependent mode of action. In the *in vitro* studies using hepatocytes from three human donors ([redacted], 2018d) CYP2B6 and CYP3A4 mRNA levels were induced by a 1.4 to 5.5-fold and a 3.8 to 6.7-fold level, respectively.

As regards induction of T4-UDP-GT (**KE2**), a slight though statistically significant increase of 16% in the activity of UDP-GT was found in F344 rats at 10,000 ppm ([redacted]i, 2002a). A statistically significant increase of 65% in the activity of UDP-GT was found at 5,000 ppm in B6C3F1 mice ([redacted], 2002b). A significant and dose-dependent induction of hepatic microsomal T4 glucuronidation activity was observed in male C57Bl/6 mice ([redacted], 2018a). The average increases were 0.3- and 1.3-fold relative to the control at dietary concentrations of 500 and 5,000 ppm, respectively. PB at a dietary concentration of 500 ppm produced on average 0.7-fold increase.

Increases in TSH plasma levels (**KE3**) were investigated in four *in vivo* studies. In rats and mice exposed to benthiavalicarb-isopropyl in the diet at 10,000 ppm and 5,000 ppm, respectively, no increase in TSH could be detected after 2 weeks ([redacted], 2002a; [redacted], 2002b). When mice were administered a diet containing 5,000 ppm of benthiavalicarb-isopropyl for up to 16 weeks a slight but statistically significant increase of 14% in TSH levels was noted ([redacted], 2003). Despite the high biological variability an increase in plasma TSH levels became apparent in mice exposed to benthiavalicarb-isopropyl in the diet at 500 and 5,000 ppm after 7 days in a 28-days *in vivo* study ([redacted], 2018a). While no increase in TSH plasma levels could be after 28 days of exposure to benthiavalicarb-isopropyl or phenobarbital, a sufficient number of changes in the pituitary gland were observed that are indicative of enhanced TSH production. The changes included an increased incidence of hypertrophic basophilic cells in the anterior pituitary and the induction of the transcription of *Trhr* and *Tshb* genes. Pituitary expression of both *Tshb* and *Trhr* genes was induced by benthiavalicarb-isopropyl in a dose-dependent manner and were only observed after 28 days of treatment at 5,000 ppm with 3.8- and 1.3-fold increases for *Tshb* and *Trhr*, respectively) PB also induced the gene transcription only after 28 days of treatment with 1.6- and 0.8-fold increases for *Tshb* and *Trhr*, respectively. Induction of the thyrotropin releasing hormone receptor (TRHR) makes the pituitary gland more sensitive for stimulation by the thyrotropin releasing hormone (TRH) from the hypothalamus in the event of a disturbance of thyroid hormone homeostasis. Induction of TSHB and the increase in the number of hypertrophic basophilic cells in the pituitary are indicative of enhanced TSH production, which should lead to increased TSH levels in the blood.

Thyroid follicular cell proliferation (**KE4**) was observed in male and female rats ([redacted], 2001), albeit there was no increase in the incidence of thyroid follicular cell tumours. 40% of the male and 50% of the female rats in the 5,000 ppm dose group and 90% of the male and 80% of the female rats in the 10,000 ppm dose groups showed thyroid follicular cell hyperplasia. In the long-term carcinogenicity study in the mouse ([redacted], 2001b), 49% of the males showed follicular cell hyperplasia at 2,500 ppm and 100% at 5,000 ppm. Benthiavalicarb-isopropyl increased thyroid follicular cell proliferation significantly *in vivo* after 28 days of treatment ([redacted], 2018a) with a 31 and 51% increase at 500 and 5,000 ppm, respectively.

Although formation of thyroid follicular cell adenomas/carcinomas (**KE5**) did not occur in male and female rats (Table 3.3-6), 40% of the males and 50% of the females at 5,000 ppm and 90% of the males and 80% of the females at 10,000 ppm were demonstrated to have thyroid follicular hyperplasia ([redacted], 2001a). In male mice a statistically significantly increased incidence of thyroid follicular cell adenoma of 18% was observed at 5,000 ppm (Table 3.3-7) with no progression to malignancy ([redacted], 2001b). 49% of the male mice showed follicular cell hyperplasia at 2,500 ppm and 100% at 5,000 ppm after 104 weeks of treatment. No statistically significantly increased incidence of follicular cell tumours was noted in female mice although there was evidence of thyroid follicular cell hyperplasia at the two highest dose levels, i.e. 50% at 2,500 ppm and 60% at 5,000 ppm. No increase in the incidence of thyroid tumours was noted after 52 weeks or 78 weeks of treatment.

Because the development of thyroid follicular cell tumours was only observed in the mouse the discussion of the Mode of Action is largely based on mechanistic studies performed in the male mouse. An overview of the results for key events from the in vivo data ([redacted]a, 2001b; [redacted], 2002b; [redacted], 2003; [redacted], 2018a) is provided in Table 3.3-8.

Table 3.3-8 *In vivo* mechanistic studies in the mouse including only statistically significant results, unless specified otherwise

dietary dose (ppm)	**KE1** Car activation, *Cyp2b10* mRNA (fold increase)	**KE2** induction of T4-UDP-GT (fold increase)	**KE3** increase in plasma TSH levels (% increase)	**KE4** follicular cell hyperplasia (incidence in %)	**KE4** follicular cell proliferation (% increase)	**KE5** follicular cell tumours (incidence in %)
500	450	0.3	slight ↗ ^{ns} (1 week)	-	31 (4 weeks)	-
2,500	-	-	-	49 (104 weeks)	-	8 ^{ns}
5,000	1,900	1.3	slight ↗ ^{ns} / 14 (1 / 16 weeks)	100 (104 weeks)	51 (4 weeks)	18

^{ns}: not statistically significant.

Although it was not possible to measure statistically significant increases in plasma TSH levels due to the large biological variability there were sufficient indications that there must have been an increased production of TSH in the pituitary by an up-regulation of the *Trhr* and *Tshb* genes and the increase in the number of hypertrophic basophilic cells in the anterior pituitary.

Concordance of dose-response relationships

- Increase in relative liver weight with dose in male mice after 1 week, 4 weeks, 13 weeks 52 weeks, 78 weeks, and 104 weeks,
- increase in the incidence of centrilobular hepatocellular hypertrophy with dose in male mice after 1 week and an increase in incidence after 13 weeks 52 weeks, 78 weeks, and 104 weeks,
- increase in *Cyp2b10* transcription with dose in male mice after 1 week,
- increase in the activity of liver T4-UDP-GT with dose after 1 week,
- increase in the transcription of *Tshb* and *Trhr* genes with dose after 28 days of treatment,
- tendency of increase in plasma TSH levels after 1 week and after 16 weeks,
- increase in follicular cell hyperplasia/cell proliferation with dose,
- increase in the incidence of follicular cell adenoma with dose in male mice after 104 weeks.

Temporal association

Key events must precede the appearance of the tumours. In the mouse, where a significant increase in tumour incidence has been observed, early key events such as up-regulation of *Cyp2b10* and *Cyp3a11* genes, increased activity of T4-UDP-GT, increased production of TSH by the pituitary, increased follicular cell hyperplasia/cell proliferation have been demonstrated to take place within 4 weeks of treatment with benthiavalicarb-isopropyl.

Strength, consistency and specificity of association of effects with key events

For the key events to be causally related to the formation of thyroid tumours, they must clearly be shown to be required steps that lead to the tumours and the findings must be reproducible. The most important early key event that is required for tumour formation is cell proliferation. In the long-term carcinogenicity study in the mouse a significant and dose-dependent increase in the incidence of follicular cell hyperplasia with dose was observed after 104 weeks of treatment. In the mechanistic study in mice a statistically significant and dose-dependent increase in the frequency of thyroid follicular cell proliferation was demonstrated after 4 weeks of treatment.

Biological plausibility

The mechanistic data that are available on the formation of follicular cell tumours in the mouse after long-term treatment with benthiavalicarb-isopropyl are consistent with a mechanism that is based on *Car*-activation, which is a MoA that has been extensively explored for phenobarbital (Elcombe *et al*., 2014). *Car*activation leads to an increase in the activity of T4-UDP-GT enhancing the excretion of thyroid hormones in the bile as their glucuronic acid conjugates. Because mice do not have thyroid hormone binding globulin a slight loss of thyroid hormone from the bloodstream through biliary

excretion is already enough to activate the hypothalamo-pituitary-thyroid (HPT) axis. The hypothalamus releases thyrotropin releasing hormone (TRH) stimulating the pituitary in producing and excreting thyroid stimulating hormone (TSH) in the bloodstream. Continuous stimulation of the thyroid by TSH produces thyroid follicular cell hypertrophy, hyperplasia and finally tumours (Hill *et al.*, 1998; Zoeller *et al.*, 2007).

Consideration of alternative Modes of Action

For benthiavalicarb-isopropyl, alternative MoAs for thyroid follicular cell carcinogenicity such as direct cytotoxicity, genotoxicity and oxidative stress have been explored in mechanistic as well as in standard toxicology studies. None of these mechanisms have been shown to be operative. Other mechanisms that can give rise to the activation of the HPT-axis are inhibition of active transport of iodide from the bloodstream into the follicular cell (Na/I-symporter), inhibition of thyroid peroxidase (TPO) converting inorganic iodide into organic iodide, inhibition of the release of thyroid hormones into the bloodstream and inhibition of the conversion of T4 to T3 by 5'-monodeiodinase in tissues. Inhibition of TPO by benthiavalicarb-isopropyl was tested *in vitro*. The result was negative such that this alternative MoA can be excluded. An overview of the results from alternative MoA studies is given in Table 3.3-9.

Table 3.3-9 Outcomes of investigations on alternative MoAs for thyroid follicular cell carcinogenicity in mice and in humans

MoA	Test	Result	Conclusion	Reference
Genotoxicity	Ames test Mouse Lymphoma Assay *In vitro* chromosomal aberration test Mouse micronucleus test	Negative. The batch (G51-08-158) that was used for the carcinogenicity studies in rats and mice showed in the Ames test a slightly positive result in *Salmonella* strain TA98 with S9. This was not confirmed in an *in vivo* gene mutation assay in transgenic mice.	not genotoxic	Mizuhashi, 2001d Nakajima, 2000a
Thyroid toxicity	13-week repeated dose study in the mouse	No histopathological changes in the thyroid up to 20,000 ppm	Not directly toxic to the thyroid.	[redacted], 1998a
	2-year carcinogenicity study in the mouse	increase in incidence of follicular cell hyperplasia in males and females at 2,500 and 5,000 ppm; increase in incidence of dilated follicles in males at 2,500 and 5,000 ppm and in females at 5,000 ppm.	Follicular hypertrophy and hyperplasia are part of the MoA and should not be seen as direct signs of toxicity.	[redacted], 2001b
Oxidative stress	Oxidative DNA damage study in mouse liver	No oxidative damage of DNA	No oxidative damage.	[redacted], 2001b
	Oral 7-day study in B6C3F1 mice at 0, 10 and 1,000 mg/kg bw, including measurement of content of *Cyp2e1* protein	No induction of *Cyp2e1* which is representative for the production of Reactive Oxygen Species (ROS).	No oxidative stress through the production of ROS.	[redacted], 2001d
Inhibition of TPO	*In vitro* assay on the inhibition of the oxidation of guaiacol	No inhibition of oxidation of guaiacol.	no inhibition of TPO.	[redacted], 2018e
Inhibition of sodium-iodide symporter	not performed	-	-	-
Inhibition 5'-monodeiodinase	not performed	-	-	-
Inhibition of release of T4/T3 in the bloodstream	not performed	-	-	-

Species differences in thyroid effects

There is a clear difference in the susceptibility to produce thyroid tumours between rats and mice. No statistically significant increase in follicular cell adenomas was recorded in the 2-year carcinogenicity study in rats whereas there was a dose-dependent and statistically significant increase in male mice, albeit there was no progression to malignancy. The difference between rodent species and humans is that in humans thyroid hormones are bound to thyroid hormone globulin, which acts as a buffer to disturbances in homeostasis of thyroid hormones. Since thyroid hormones in rodent species do not have this buffer function, rodents are much more sensitive to changes in thyroid hormone levels. Small changes in blood thyroid hormone levels will activate the HPT-axis with an increased production of TSH.

Is the weight of evidence sufficient to establish the mode of action in animals?

There is sufficient weight of evidence. It has been demonstrated that the MoA of benthiavalicarb-isopropyl underlying the production of thyroid follicular cell tumours in the mouse is based on Car activation and is compatible with the MoA described for rodent thyroid follicular cell tumours induced by phenobarbital. The relevant molecular and pathological endpoints for benthiavalicarb-isopropyl-induced thyroid effects in mice are supported by repeated dose standard toxicity and mechanistic studies in mice. There is a good dose-response relationship of the data on the key events of the MoA for the follicular cell tumours. **KE1** of the MoA is defined as activation of the Car nuclear receptor, which was measured by induction of *Cyp2b10* gene transcription and associated liver enzyme activity. **KE2** is the increase in activity of T4-UDPGT leading to enhanced conjugation of thyroid hormones with glucuronic acid and excretion via the bile. A dose-dependent increase in the activity of T4-UDPGT has been demonstrated in three *in vivo* studies. **KE3** is related to the enhanced production of TSH by the pituitary. A tendency of an increase in plasma TSH levels was noted after 1 week and after 16 weeks. Although it was not possible to measure a statistically significant increase in TSH plasma levels in mice after 7 and 28 days of treatment because of a too high inter-animal variability, there are sufficient changes in the pituitary supporting an early increase of the biosynthesis of TSH, such as enhanced transcription of *Trhr* and *Tshb* genes and an increased number of hypertrophic basal cells in the anterior pituitary. **KE4** is the increase in thyroid follicular cell proliferation. An increase in the incidence of follicular cell hyperplasia was evident in the carcinogenicity test in mice after 104 weeks. An increase in the frequency of thyroid follicular cell proliferation was observed in mice after 28 days of treatment. Considering all the relevant mechanistic and standard toxicity studies, the key events show clear changes that are consistent with a Car-mediated MoA leading to thyroid follicular cell hyperplasia and ultimately adenomas and carcinomas. In addition, other possible MoAs, in particular inhibition of TPO, were investigated and were found not to be operative.

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

Human relevance of the MoA can be exluded. Benthiavalicarb-isopropyl has been shown to induce the activity of T4-UDP-GT through Car activation in mice. The enhanced elimination of thyroid hormones through biliary excretion of their glucuronic acid conjugates activates the HPT axis to restore homeostasis. Both humans and rodents have non-specific low affinity protein carriers of thyroid hormones, *e.g*. albumin and α1-acid glycoprotein. However, humans as well as primates and dogs have a high affinity binding protein, *i.e*. thyroxine binding globulin, which binds T4 and to a lesser extent T3. This protein is absent in rodents and other lower vertebrates, which makes these species much more sensitive to the elimination of thyroid hormones from the bloodstream. Besides the absence of a specific binding globulin, the half-life of T4 in rats (0.5 to 1 day) is much shorter than in human (5 to 9 days). This difference in half-lives results in a 10-fold greater requirement for endogenous T4 in the rat thyroid than in adult humans. TSH serum levels in rats are also 6 to 60 times higher than in humans (Hill *et al*., 1998).

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

As human relevance of the experimental animal MoA can be reasonably excluded on the basis of qualitative differences in key events, a quantitative assessment of kinetic or dynamic factors is not deemed necessary. The key events of the MoA of the tumour formation in mice and its relevance to man following the principles of the human relevance framework (Cohen *et al*., 2003; Cohen *et al*., 2004) are summarized in Table 3.3-10.

Table 3.3-10 Key events and other related events as part of the overall MoA of benthiavalicarb-isopropyl in mice and humans.

key and related events	evidence		relevance for humans
	mice *in vivo*	humans *in vitro*	
Cyp2b10 mRNA induction (Car activation)	yes	yes (CYP2B6)	likely
T4-UDP-GT induction	yes	not	likely
Presence of thyroxine binding globulin	no	np	yes
Increase in TSH production	yes	np	unlikely
Thyroid follicular cell proliferation	yes	np	unlikely
Thyroid follicular adenoma/carcinoma	yes	np	unlikely

np: not performed

From the data of standard toxicity and mechanistic toxicology studies *in vitro* and *in vivo* it can be concluded that the thyroid follicular cell tumours produced by benthiavalicarb-isopropyl in male mice are based on a mode of action which operates through Car activation. The key events being (1) Car activation, (2) increased activity of T4 UDP-GT, (3) increased production of TSH by the pituitary, (3) thyroid follicular cell proliferation and (4) thyroid follicular cell adenomas that are typical for this MoA in mice have been demonstrated to be present. The concordance of dose-response relationships, the temporal association, the strength, the consistency and specificity of association with the tumour response, the biological plausibility, the absence of alternative MoAs and the species specificity of these key events have been proven. Therefore, benthiavalicarb-isopropyl can be considered to be a compound acting through a Car-mediated MoA similar to that of phenobarbital, which is considered not to be relevant to humans (Meek *et al*, 2003; Elcombe *et al*., 2014). Consequently, benthiavalicarb-isopropyl is not expected to have an adverse effect on the HPT axis (T-modality) in humans.

3.3.2.3 **Non-EATS modalities**

Effects on adrenal weight and histopathology were observed in sub-chronic, chronic, reproductive and developmental toxicity studies with mice, rats and dogs ([redacted], 2001a; [redacted], 2001b; [redacted], 1998a; [redacted], 1998b; [redacted], 1999; [redacted], 1999; [redacted], 2000a; [redacted], 2004).

There was no effect on absolute adrenal weight in male and female mice after a 13-weeks treatment with benthiavalicarb-isopropyl. A statistically significant increase in relative adrenal weight was however observed in males at 7,000 and 20,000 ppm. There was no effect on relative adrenal weight in the female mice. The effect on relative adrenal weight in males was associated with body weight loss ([redacted], 1998a). A statistically significant increase in absolute adrenal weight in male and female rats and in relative adrenal weight in male rats was observed at 5,000 and 20,000 ppm after a 13-weeks treatment ([redacted], 1998b). Statistically significant increase in absolute adrenal weight was observed in male mice at 100, 2,500 and 5,000 ppm but without any dose/effect relationship after 104 weeks. There were no effects on absolute adrenal weight in female mice at any observation time and in male mice at weeks 52 and 78. Statistically significant increase in relative adrenal weight in male mice were observed at 2,500 and 5,000 ppm after 52 weeks, at 5,000 ppm after 78 weeks and at 2,500 and 5,000 ppm after 104 weeks, albeit there was no clear dose/effect relationship and the relative weight changes were associated with body weight loss. There were no effects on relative adrenal weight in female mice ([redacted], 2001b). Slight and inconsistent though statistically significant increases in absolute and relative adrenal weight in male and female rats were observed at 5,000 and

10,000 ppm at various time points up to 104 weeks but without a clear dose effect relationship ([redacted], 2001a). Statistically significant increases in absolute adrenal weight were observed in female dogs at 200 and 1,000 mg/kg bw/day. An increase in relative adrenal weight was also observed in females at 200 mg/kg bw/day after 13 weeks of treatment ([redacted], 1999). In the two-generation reproductive toxicity study a statistically significant increase in absolute and relative adrenal weight was observed in male rats of the F0 generation at 10,000 ppm ([redacted]a, 1999). There was no substance related increase in absolute and relative adrenal weight in females of the F0 generation and males and females of the F1 generation. In two embryo-foetal developmental toxicity studies there were significant increases in absolute and relative adrenal weight in the dams at dose levels of 100 and 1,000 mg/kg bw/day ([redacted], 2000a; [redacted], 2004).

An increase in absolute and/or relative adrenal weight was observed in sub-chronic and chronic studies with mice, rats and dogs, in a multi-generation rat reproductive toxicity study and in two embryo-foetal development toxicology studies with rats. Changes in adrenal weight are common in toxicity studies at dose levels that produce toxicity and stress to the animals. The only study with changes in the histopathology of the adrenals is the chronic study with mice ([redacted] 2001b), where a clear and statistically significant increase in the incidence of adrenal cortex hypertrophy was noted in males and females at 2,500 and 5,000 ppm after 104 weeks of treatment. There was however no response at the lower tested dose levels. Dose levels of 2,500 and 5,000 ppm caused excessive toxicity, which may have stimulated corticoid biosynthesis due to stress. In the 13-week toxicity study with mice ([redacted], 1998a) no substance related effects were seen in the adrenal cortex at dietary concentrations up to 20,000 ppm. If benthiavalicarb-isopropyl would have an adverse effect on the hypothalamo-pituitary-adrenal (HPA) axis, then adrenal cortical hypertrophy with vacuolation in the *zona fasciculata* should have been prominent after 13 weeks of exposure. In conclusion, changes in absolute and relative weight of the adrenals have been observed in various studies but often without a clear dose related effect and without a histopathological correlate, i.e. hypertrophy of the adrenal cortex. The only exception is the 2-year carcinogenicity study with mice at dose levels above the MTD. In the mouse 13-week toxicity study no histopathological changes, i.e. vacuolation in cells of the *zona fasciculate*, were observed in the adrenal cortex, which indicates that benthiavalicarb-isopropyl has no adverse effect on the HPA axis. Consequently, the effects seen on the adrenals are secondary to stress produced by excessive toxicity and body weight loss.

3.4 Overall conclusion

All standard toxicity and mechanistic studies have been thoroughly analysed for possible effects on the endocrine system, including EATS and non-EATS modalities, and the results are assessmbled in a table with lines of evidence for the EAS and T modalities. All possible effects on apical endpoints with a possible endocrine MoA were examined and found to be negative.
The points discussed include (1) the increase in estradiol serum levels in the 2-year carcinogenicity study with rats ([redacted], 2001a), (2) the increased incidence of uterine adenocarcinoma in rats ([redacted], 2001a), (3) the effects on ovary weight and histopathology in mice ([redacted], 2001b; [redacted], 1998a), (4) the effects on prostate weight and histopathology in dogs ([redacted], 1999), (5) the effect on testes weight in rats ([redacted], 2001a); (6) the effects on sperm count in rats ([redacted], 1999) and (7) the effects on pituitary histopathology ([redacted], 2001b, [redacted], 2001). All points are thoroughly assessed and were found to be not related to an endocrine disruptive mode of action. Moreover, it needs to be emphasized that all of the aforementioned effects were observed at dose levels, which produced excessive toxicity and should for that reason not have been taken into account for the assessment of endocrine disruption properties. On the basis of all the available toxiciological data, it can be concluded without any reasonable doubt that benthiavalicarb-isopropyl is not an endocrine disruptor in humans.

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5 COMMENTS ON EFSA'S ED ASSESSMENT

5.1 Inconsistencies between EFSA's statements and available study data

The EFSA made several statements during the two Pesticide Peer Review expert meetings which can be verified as incorrect. These are highlighted in the below table. It is a non-exhaustive list only covering major oversights. It is of note that the answer to these oversights are devoid of scientific interpretation.

EFSA statement (from meeting minutes of the Experts' Meeting 186 or Experts' TC 203)	Applicant's answer to oversights and/or flaws (no
About the carcinogenicity study in rats (Murata 2001a): "In vivo mechanistic: hormonal changes in estradiol (increase) on weeks 26, 78 and 104 in the carcinogenicity study in the rat at ≥5000 ppm; no measurement of FSH, prolactin or progesterone in the carcinogenicity study. […]" (Experts' Meeting 186)	In the carcinogenicity study in rats (Murata 2001a): At 104 weeks there was no change in oestradiol levels in any of the tested doses. Furthermore, progesterone levels were measured in the long term carcinogenicity study in rats. The progesterone levels and the oestradiol to progesterone ratio were both evaluated and remained unchanged at all sampling time and all tested doses.
"EATS mediated parameters: uterus histopathology in 104 weeks in rat below MTD and ovary histopathology in mouse (concordance with decrease corpora lutea and weight of uterus)". (Experts' Meeting 186)	In the carcinogenicity study in rats (Murata 2001a): effect on uterus (see above) but no effect in ovary (weight + histopathology). In the mouse studies (carcinogenicity studies Murata 2001b and 13 weeks sub-chronic toxicity Inoue 1998a): there was no effect on the uterus (weight or histopathology). Concordance of ovary weight with decrease *corpora lutea* (i.e. observed in the same individuals) was only noted in the 13 weeks study at 13week at 20,000 ppm at excessive toxicity (high decrease weight gain in males, liver necrosis in males and females…)
About postulated mode of actions for uterine adenocarcinoma: "continuous increase in E2 to P4 ratio (senescence or chemically induce imbalance in sex steroid hormones altering the E2 to P4 ratio). Hormonal changes independent from estrogen receptors signalling is considered a later event in rodents and human endometrial type of cancer: possible" (Experts' Meeting 186)	Two studies have measured the E_2 /P_4 ratio which remained unchanged in both of them at any doses and sampling time (long term carcinogenicity study in rats Murata 2001a and 8 weeks study in female rats for hormones measurement and aromatase activity, Inagaki 2002c). This cannot be a possible mechanism.
"[…] (no data on estradiol clearance, estradiol metabolites, CYP specific induction e.g. CYP1B, and measurement of oestrus-cycle dependent hormones in line with acknowledgment of oestrus-cycle period were provided)".	In the 8 weeks study (Inagaki 2002c) both oestradiol and progesterone were measured. It was specified in the study report that this was done at diestrus while in the long term carcinogenicity study (Murata 2001a), this information was not available. Possible modulation of oestrogen metabolism through activation of the AhR receptor and subsequent induction of CYP1A1, CYP1A2, and CYP1B1 enzymes in the liver was addressed in vivo studies in rats and mice (Murata 2001c and 2001d)
"a more thorough analysis to substantiate lack of pre-neoplastic changes should have been provided e.g. by considering the pathology of uterus in the sensitive rat strain for uterine adenocarcinoma (*e.g.* Donryu rats)".	In the Murata 2001a study report information is provided on this point with the associated reference. For further information, reference is made to Section 3.3.2.1.
"For the uterine and ovary effects which are considered EAS mediated adverse effects and for which a concomitant increase in circulating levels of estradiol were observed in the rat, […]"	Effect in ovary were never observed in the rats in any of the conducted studies (Inoue, 1998b; Murata, 2001a; Tanaka, 1999, Inagaki 2002c)
"Moreover, EFSA commented that no evaluation of the oestrus cycle was reported for any study".	This parameter was available and submitted since the beginning of the dossier in the two generation rats study (Tanaka, 1999)

5.2 Applicant's statement regarding EFSA's scientific assessment and interpretation of the ED GD

The EFSA defended during the 9 January 2019 Peer Review Meeting that the oestradiol increase seen in the long term study in rats cannot be dismissed and that the most likely cause of the uterine adenocarcinoma is an underlying endocrine activity of the active substance. This viewpoint led to the conclusion that criteria are met for an endocrine disruptor in humans.

Although the guidance allows that it "may be sufficient to assess the link and come to a conclusion on the biological plausibility between adverse effects and the endocrine activity", the existing scientific knowledge regarding the uterine adenocarcinoma does not provide evidence of a biological link between the sole estradiol increase in the rat carcinogenicity and the observed uterine adenocarcinoma. It is not the increase in estradiol levels, as stated by the EFSA, but the increase of the E_2/P_4-ratio that is the key parameter for the formation of uterine adenocarcinoma (see Section 3). There is no other evidence, which is indicative of benthiavalicarb-isopropyl having endocrine disrupting properties. As stated in Section 1 and Section 4.1 of this document, not all available parameters and data were taken into account in EFSA's assessment. Furthermore, the toxicological data and existing scientific literature indicates that the uterine adenocarcinoma caused by the administration of high doses of benthiavalicarb-isopropyl is not *per se* a consequence of an endocrine mode of action thereby not all criteria for an endocrine disrupting substance as laid down in Regulation (EU) No 2018/605 are met.

In particular, one pivotal mechanistic study was considered by the EFSA as unreliable during the second expert consultation although its validity had never been questioned before. Additionally, no substantive criticisms were given but only a reference to Appendix B of the ED GD was made. According to Appendix B of the ED GD giving recommendations for the design, conduct and technical evaluation of hormonal studies, the invalidated study does provide all necessary information on animals, number, age, sex, care, oestrous-phase sampling time, blood sampling, euthanasia, sample collection and storage, statistical analyses and methods of analysis. With regard to the appropriateness of the method used for measuring aromatase activity and hormone levels, the measurements were made using commercially available kits that can be considered sufficiently accurate and precise. Additional validations by the performing laboratory for estradiol, progesterone, T3, T4 and testosterone increase the confidence in the results obtained. The results are provided in Annexes 5 and 6 of this document. Additionally, in the rat carcinogenicity study no information was available on the oestrous phase at which hormone measurements were made while in the mechanistic study, it is specified that all hormone measurements were made at di-oestrous phase.

While it was stated that there is no information available on oestrous cyclicity, this information is available in a two-generation toxicity study in rats. Furthermore, no endocrine adversity was observed in the parent animals or their progeny, which strengthens the fact that benthiavalicarb-isopropyl is not an endocrine disruptor. Although at this time no specific mode of action could be evidenced for the uterine adenocarcinoma, which should be discussed in the context of the carcinogenicity, it is very likely the uterine adenocarcinoma and the other observed tumour types are the consequence of excessive toxicity in the experimental animals.

Overall, the toxicological data package and the holistic analysis of the integrated lines of evidence does not provide any clear indication of endocrine disrupting properties for benthiavalicarb-isopropyl for humans.