Isobutyl paraben, CAS no. 4247-02-3

Synonyms: Isobutyl 4-hydroxybenzoate, isobutyl-p-hydroxybenzoate, isobutyl parahydroxybenzoate, 2-methylpropyl 4-hydroxybenzoate, IBP

Isobutyl paraben ($C_{11}H_{14}O_3$) (Figure 1) is a nonlinear paraben (Figure 1). Parabens are a group of alkyl esters of p-hydroxybenzoic acid, and many different parabens exist, e.g. methylparaben, ethylparaben and butylparaben. Isobutyl parabens is used as preservatives in foods, pharmaceuticals and cosmetics and is used in 0-10 tonnes per annum. Isobutyl paraben has been classified in ECHA as a skin sensitiser 1B (H317: May cause an allergic skin reaction). Moreover, it has been classified as a skin (H315: causes skin irritation) and eye irritant (H318: causes serious eye damage and H319: causes serious eye irritation)

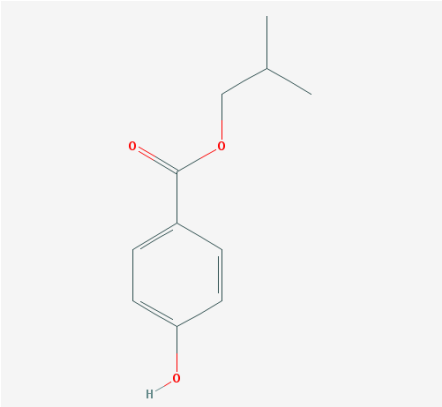

Figure 1: 2D structure from PubChem

4. Human health hazard assessment

4.10.3 Endocrine disruption

4.10.3.1 General approach – human health

4.10.3.2 In vitro information indicative of endocrine activity

Kim et al. (2012)

Summary: The aim of the study was to investigate the additive, synergistic or antagonistic effects on estrogenic activity of isobutyl paraben and octylphenol using GH3 rat pituitary cells. Isobutyl paraben was tested at 0, 0.1, 1 or 10 µM and gene expression and protein expression of Calbindin-D_{9k} (CaBP-9*k*) and progesterone receptor (PR) and estrogen receptors (ER) reporter gene expression was assessed in the cells. Expression of the ER reporter gene and expression of CaBP-9*k* gene and protein was upregulated at all tested doses of isobutyl paraben. The PR gene was also upregulated in all the tested doses of isobutylparaben but only the highest dose (10 µM) upregulated expression of PR protein.

Study quality and assessment: The study is well-described and thorough. The methods section describes the concentrations of isobutyl paraben tested and the incubation conditions and they used positive and negative controls and performed duplicate or triplicate samples. Information on cytotoxicity was apparently not tested and the CAS-number of the chemicals used is not provided. Based on this, the study is assessed to be of medium quality. The study provides moderate evidence of an estrogenic mode of action of isobutyl paraben involving estrogen receptors.

Kim et al. (2011)
Summary: The objective of this study was to validate the estrogenic activity of a range of chemicals, including parabens, using the stably transfected transcriptional activation assay. Isobutyl paraben was tested in a hERα-HeLa-9903 cell line transfected with a human ERα gene with a firefly luciferase gene as a reporter gene. The study showed (22,000 fold) lower estrogenic activity of isobutyl paraben compared to 17β-estradiol. Compared to other parabens tested, isobutyl and butyl paraben were the two parabens with highest estrogenic activity.

Study quality and assessment: The study is well-described and well-structured and it provides much information on the cell assay performed such as positive and negative controls, incubation conditions, dilutions etc. However, information on cytotoxicity or triplicates is not described and no CAS number or purity of isobutyl paraben is given. The study is therefore assessed to be of medium quality. The present study investigated the activity of isobutyl paraben in a single assay and the resulting information on the estrogenic mode of action of isobutyl paraben is limited. The data provide weak evidence of an estrogenic mode of action of isobutyl paraben

Kim et al. (2010)
Summary: In this study, the aim was to determine the relative androgen receptor (AR) binding affinity of a range of chemicals, e.g. parabens, phthalates and testosterone. Isobutyl paraben showed higher competitive affinity to AR than other parabens tested. The binding affinity relative to dihydrotestosterone was almost 17000 times lower for isobutyl paraben.

Study quality and assessment: The description of the methods and the interpretation of the findings are poor. The CAS-numbers of chemicals or of the radioligand are not given and controls are not used. The concentrations tested can be read with some difficulty from a figure of low graphical quality. Technical notes for the user in the laboratory are included in the description of the methods giving the impression that the materials and methods section is not written with the reader of the article in mind. However, other details are included such as a description of the AR used, a description of the preparation of the assay buffer and the experiment was performed in triplicates. The study is assessed to be of medium quality. This study investigated the relative binding affinity to AR but does not investigate other antiandrogenic mechanisms of action and the knowledge provided on the antiandrogenic properties of isobutyl paraben are limited. The data provide weak evidence of an antiandrogenic mode of action of isobutyl paraben.

Vo et al. (2010)
Summary: In this study a competitive ligand binding assay was performed in addition to a female pubertal assay to assess the estrogenic effects of several parabens *in vitro* and in female rats. The binding affinity of isobutyl paraben to ERα and ERβ was investigated. Isobutyl paraben showed affinity to both receptors and no preference to any of the receptors could be determined.

Study quality and assessment: The *in* vitro study is described very briefly and it is not mentioned where the receptors used in the assay originate from (e.g. rat or human) or whether triplicates were performed. The quality of the *in vitro* part of the study is assessed to be of low quality. The study provides weak evidence of an estrogenic mode of action of isobutyl paraben.

Dabre et al. (2002)
Summary: This study investigated the estrogenic activity of isobutyl paraben and n-butyl paraben in an array of *in vivo* and *in vitro* assays. *In vitro* studies were performed in three different human breast cancer cell lines. A competitive ERα binding assay in and the ability to regulate gene expression oestrogen responsive genes were performed in MCF-7 cells. Oestrogen dependent growth was assessed in MCF-7 and ZR-75-1 cells lines and MDA-MB-231 cells were used as a negative control.

Isobutyl paraben increased expression of oestrogen regulated genes, stimulated oestrogen dependent growth of cell lines and inhibited binding of oestradiol to ERα. These *in vitro* data support each other and altogether they indicate an estrogenic activity of isobutyl paraben at receptor, gene and at a cellular physiologic response level.

Study quality and assessment: The study is well-described and much information is included for the *in vitro* part of the study, such as source of cell lines, use of negative and positive controls and clear descriptions of the interpretation and objective of each study. Although the CAS number and purity of isobutyl paraben is not reported, the *in vitro* study is assessed to be of high quality. The *in vitro* studies investigated the estrogenic activity of isobutyl paraben at several levels, including at receptor, at gene and at a cellular physiologic response level. The *in vitro* part of the study provides strong evidence of an estrogenic mode of action of isobutyl paraben.

Okubo et al. (2001)
Summary: In the present study the estrogenic activity of parabens was investigated *in vitro*. MCF-7 cells were used to study estrogenic induced cellular proliferation, gene expression levels of ERα and PR and the protein expression of ERα. A competitive receptor binding assay was performed to investigate the affinity to human ERα and ERβ.

Proliferation of MCF-7 cells was increased by isobutyl paraben. Compared to the positive control, 17β-estradiol, the effect of isobutyl paraben was 170,000 times lower. Suppression of the proliferative effect by the antiestrogen ICI 182,780 interacting with ER, suggested an estrogenic mode of action of isobutyl paraben on proliferation of MCF-7 cells. Gene expression of ERα was decreased and PR was increased by isobutyl paraben. Although the effects were lower than the effects of 17β-estradiol, gene expression of ERα and PR were changed in the same directions as for 17β-estradiol. Protein expression of ERα was decreased by isobutyl paraben although not in the same extent as for 17β-estradiol. This is in good accordance with the findings on gene expression level of ERα. Isobutyl paraben had 1000-fold lower affinity to human ERα and ERβ compared to the positive control DES, indicating that the paraben does not have specific preference for ERα or ERβ.

Study quality and assessment: The study is well-described and thorough. Although Okubo and co-workers did not mention whether they assessed cytotoxicity, this does not affect the reliability of the study as the results indirectly imply that there was no cytotoxicity at the relevant doses. Proliferation was observed and proliferation levels were not suppressed below control levels at dilutions of ICI 182,780 below 10^{-8}. Although the CAS-number and purity of isobutyl paraben is not described, the study is assessed to be of high quality. Based on the data on estrogenic activity of isobutyl paraben at the gene, protein and cellular proliferation levels together with the study on affinity to human oestrogen receptors, the study provides strong evidence of an estrogenic mode of action of isobutyl paraben.

4.10.3.3 In vivo effects with regard to an endocrine mode of action

Yang et al. (2016)

Summary: The aim was to investigate reproductive effects in male rats of a low dose of a mixture of bisphenol A and isobutyl paraben after developmental exposure. The two compounds were tested separately and in a mixture. Pregnant rat dams (n=3/group) were dosed by oral gavage with 2.5 mg/kg/day isobutyl paraben from gestation day 6 to post-natal day 12. At delivery, birth weight, number of pups and the sex ratio were recorded. Male pups were assessed for anogenital distance (AGD), nipple retention, preputial separation, testis descent, body weight, pinna detachment, incisor eruption and eye opening. The litters were adjusted to 8 male pups per group from PND4. Male pups were killed on PND70 and blood was sampled for hormone analysis (LH, FSH, testosterone and 17β-estradiol), testis and epididymis were weighed and stored for histopathological analysis and epididymal sperm count and motility (n=5/group) was assessed. The hormone level of 17β-estradiol was decreased by isobutyl paraben in adult male pups. Sperm count and motility was reduced compared to controls in adult male offspring in the isobutyl paraben group. No other endpoints investigated were affected by isobutyl paraben.

Study quality and assessment: The study is well described and although the chow was not tested for phytoestrogen content, oestrogen contamination was minimized from other sources (stainless steel cage, glass water bottles and wood bedding). Only 3 pregnant dams were used for this developmental study and data from littermates were analysed but the litter effect was not included in the statistical analysis. The study is assessed to be of low to medium quality. This study showed adverse effects on sperm motility and sperm numbers, but the underlying mode of action is not clear. Effects on sperm count have been seen for several endocrine disrupters after developmental exposure suggesting an endocrine mode of action of isobutyl paraben. Moreover, the study showed effects on the intrinsic oestrogen levels indicating an endocrine mode of action of isobutyl paraben *in vivo*. The study provides weak evidence of an endocrine mode of action and adverse effects of isobutyl paraben.

Kim et al. (2015)

Summary: In this study, a 28-day repeated dose toxicity study with dermal exposure to isobutyl paraben, isopropyl paraben or a mixture of the two was performed. The test followed the OECD test guideline 410. Male and female rats (5 weeks old) were dermally exposed to 0, 100, 300 or 600 mg/kg isobutyl paraben (10 rats per group) for 28 days (5 days per week) by topical application to the skin in a shaved dorsal area of the trunk. Body weight and food and water consumption was measured. At necropsy, blood was sampled (for haematological parameters, biochemical parameters and hormone analysis of T3, FSH, estradiol, testosterone and insulin) and livers, kidneys, hearts, brains, testes, prostates, ovaries and vaginas were weighed. Skin, liver, kidney brain and heart were assessed histopathologically. Histopathological evaluation of the skin showed changes in the females. Epidermal hyperplasia with hyperkeratosis (1 out of 3 examined in the 100mg/kg dose-group and 3/3 in the 300 and 600 mg/kg groups) and pustules (1/3 in the 100 and 300 mg/kg dose-groups and 3/3 in the 600 mg/kg dose-group) were seen. Biochemical parameters measured in the blood showed increased levels of Na^+ in a dose-dependent manner in males from 50mg/kg and higher and increased levels of Cl^- in females from the 600 mg/kg group compared to controls; however these changes were within the normal range. The lack of changes in organ weights and haematological parameters indicated no signs of heamatotoxic, hepatotoxic or nephrotoxic effects of isobutyl paraben in the tested doses.

Study quality and assessment: The study is well-described and thorough and follows the OECD TG 410 for repeated dose dermal toxicity. It is unclear how many male and female rats were used in the study (5 or 10 of each sex per group), and a little more details on the housing conditions would have been preferred. The study is assessed to be of medium quality. Due to the lack of effects on endocrine-related organs and hormonal parameters, the study provides no evidence of adverse effects with an endocrine mode of action.

Kawaguchi et al. (2010)

Summary: In this study the effects of perinatal exposure to isobutyl paraben on social behaviour was analysed. Pregnant rat dams were continuously exposed to isobutyl paraben sc. through a Silastic capsule implanted 3 weeks before mating. Female offspring (n=5-6) were tested for social recognition at 16 weeks of age.

Treated rats showed impaired social recognition. After repeated presentation to a rat, females exposed to isobutyl paraben spent the same time interacting with the rat compared to the first presentation, whereas control females spent less time interacting with the rat on the 4th trial.

Study quality and assessment: The study is well-described and relevant information is given. The study is assessed to be of high quality. The study provides evidence of effects on social behaviour, but it is not clear if the adverse effects are due to an endocrine mode of action. This study provides strong evidence of adverse effects.

Vo et al. (2010)

Summary: In this study a female pubertal assay was performed to investigate the effects of parabens (methyl-, ethyl-, propyl-, isopropyl- butyl- and isobutyl paraben) in female rats and an affinity ligand binding assay was performed to assess the estrogenic activity *in vitro*. Female prepubertal rats (10/group) were dosed PND21-40 with 0, 62.5, 250 or 1000 mg/kg/day isobutyl paraben by oral gavage. 17α-ethynylestradiol was used as an estrogenic positive control. Oestrous cycle was assessed during the exposure period. At necropsy on PND41 blood was sampled for hormone analysis (estradiol, prolactin, tetra-iodothyronine (T4) and TSH) and organs (uterus, ovary, liver, kidneys, adrenal glands and thyroid glands) were weighed and stored for histopathology.

At all doses of isobutyl paraben myometrial hyperplasia was seen in uteri. It is less clear what changes were observed histologically in ovaries, but a decreased number of corpora lutea, increased number of cystic follicles and thinning of the follicular epithelium was apparently seen. Serum levels of T4 were decreased in the low-dose isobutyl paraben group but not at higher doses.

Study quality and assessment: The present study has some limitations and missing information. Although animals were fed a soy-free diet to limit contamination of phytoestrogens through the feed, other sources of oestrogen contamination such as cage material or drinking bottles were not avoided. The rats were bred in-house but it is not described whether some of the 200 female prepubertal rats used in the study were littermates. The description of histopathological findings in ovaries is confusing and it is unclear what kind of changes they found in this organ. Vaginal opening was assessed, but is not described in the materials and methods section. Finally, the relevance of assessing the regularity of the oestrous cycle in vaginal smears from PND21 is not clear as the female rats are not sexually mature at this age and the mean day of vaginal opening in some of the exposure groups

was above 36 meaning that they had less than 4 days to assess vaginal smears in some of the animals in those groups. As a consequence, the study is assessed to be of low quality. The histopathological changes observed in female reproductive organs may be related to estrogenic effects of isobutyl paraben, but the underlying mechanism of action is not clear. The role of isobutyl paraben on thyroid hormones is unclear and the changes in T4 levels may be a chance finding. The study provides weak evidence of adverse effects in female reproductive system with an estrogenic mode of action of isobutyl paraben. Also, a weak evidence of a thyroid disrupting mode of action of isobutyl paraben is provided based on the hormonal effects seen at the lowest dose.

Kawaguchi et al. (2009a)

Summary: In this study, the effects of isobutyl paraben on emotional behaviour and learning performance were analysed in rats. From 3 weeks before mating, mated dams were exposed continuously to isobutyl paraben through an implanted Silastic capsule. Offspring were tested for neurobehavioral function. In the open field test, 5 weeks old offspring (one male and one female per litter from 7 treated and 8 control litters) were tested. The same animals were tested in the elevated plus maze at 6 weeks of age. In the passive avoidance test, females were ovarectomised (ovx) at 7 weeks of age and treated with or without oestrogen and 11 weeks old males and ovx females with or without oestrogen from 8 treated and 8 control dams (one of each per litter) were tested. The same animals were used in the Morris water maze. The amount of isobutyl paraben released from the Silastic capsule was estimated by incubation of capsules in saline at 37°C for 42 days.

Approximately 4.36 mg/L/day of isobutyl paraben was released from the capsule *in vitro*. Behavioural testing showed sex-specific effects. Male offspring exposed to isobutyl paraben perinatally spent shorter time in the open arms of the elevated plus maze and showed decreased performance in the passive avoidance test. No effects of isobutyl paraben on offspring behaviour were seen in the open field or the Morris water maze. Developmental exposure to isobutyl paraben affected emotionality but not activity levels. Long-lasting effects on behaviour appeared to be present in male rats after developmental exposure to isobutyl paraben and the anxiogenic affects observed in male offspring may be comparable to behavioural effects seen for gonadal hormones or compounds that mimic oestrogen. The results indicate that the estrogenic action of isobutyl paraben shown in the literature may feminise the male brain.

Study quality and assessment: This study is well-described and the relevant information is given in the text. Although no CAS-number of the compound is reported, the study is assessed to be of high quality. The adverse effects on male behaviour are in good accordance with the estrogenic activity of isobutyl paraben reported by others and this study provides strong evidence of adverse effects linked to an estrogenic mode of action of isobutyl paraben.

Kawaguchi et al. (2009b)

Summary: The aim was to clarify the estrogenic effects of gestational and through lactation exposure to isobutyl paraben on the endocrine system of pregnant dams and offspring. This study was the same as for Kawaguchi et al. (2009a), but with assessments of other effects. Pregnant rat dams were continuously exposed to isobutyl paraben sc. through a Silastic capsule. Dams were killed after weaning and blood was collected and pituitary glands, adrenal glands and uterus were weighed. AGD was measured in 7 days old offspring (4 males and 4 females per litter) and vaginal opening and oestrous cycle from 7 weeks of age were assessed in female offspring (1 female per litter). Offspring

were killed at 3 weeks of age (1 of each sex per litter) and at 12 weeks of age (1 male per litter). At necropsy of offspring blood was collected and pituitary glands, adrenal glands, testis and uterus were weighed. One testis from 12 weeks old male offspring was used for sperm count. The 12 weeks old males were stressed before necropsy and adrenal glands were collected. One 7 weeks old female per litter was ovariectomised and implanted with Silastic capsules with or without estradiol. At 12 weeks of age (5 weeks after implantation of the capsules) female offspring treated with estradiol were killed and blood was collected and pituitary glands and uterus were weighed. Blood samples from ovariectomised females not treated with estradiol were collected at 13 and 17 weeks of age. Blood was analysed for hormone levels (LH, FSH, testosterone, estradiol, prolactin, ir-inhibin, TT3, TT4 and corticosterone).

No overt signs of toxicity were seen. There were no effects on litter size or ratio of male pups. Plasma corticosterone levels in dams were decreased after isobutyl paraben exposure and uterus weight was increased. No effects were found in the offspring.

Study quality and assessment: The study is well-described and all relevant information is given in the text except for the CAS number of the tested isobutyl paraben. The study is assessed to be of high quality. The data from the study show evidence of effects of isobutyl paraben on uterus weights of dams but not in female offspring. The study provides weak evidence of adverse effects related to an estrogenic mode of action and no evidence an antiandrogenic or a thyroid disrupting mode of action.

Koda et al. (2005)

Summary: The objective of the study was to test the oestrogenic activity of the agents in UV filters, including isobutyl paraben, using ethinyl estradiol and bisphenol A (BPA) as positive controls in a uterotrophic assay. Ovariectomised female rats (13-14 weeks old at study start, n=6) were dosed s.c. daily for 3 days with 0, 100, 250 or 625 mg/kg/day. Body weight, wet and blotted uterine weight was assessed. Wet and blotted uterine weights were increased with 250 mg/kg isobutyl paraben per day or more, indicating an estrogenic activity of the chemical *in vivo*.

Study quality and assessment: The study is well-described, the estrognic activity of the feed was tested and aluminium cages and water supply without plastic were used to minimize contamination with BPA. The study is assessed to be of high quality. This study provides strong evidence of an estrogenic mode of action of butyl paraben *in vivo*.

Dabre et al. (2002)

Summary: This study investigates the estrogenic activity of isobutyl paraben and n-butyl paraben in an array of *in vivo* and *in vitro* assays. The immature rodent uterotrophic assay was used in the *in vivo* study. Immature (18 days of age) female CD1 mice (7 per group) were dosed s.c. with 0, 1.2 or 12 mg/kg/day for 3 days. Body weight and uterus wet weight were assessed at necropsy.

Isobutyl paraben increased uterus weight relative to body weight at 1.2 and 12 mg/kg/day. The results indicate an estrogenic activity of isobutyl paraben *in vivo*.

Study quality and assessment: In the *in vivo* part of the study, information on housing conditions is missing. In a study with focus on oestrogen activity of a compound like in the present study, some information on (phyto)oestrogen content in the feed, cage material or cage enrichment would be

preferred. However, in the present case with a very short exposure period (3 days), this information is less critical and the remaining work is very well described. Although CAS-number and purity of the compound is not reported, the quality of the *in vivo* study is assessed to be high. The *in vivo* data show an estrogenic mode of action of isobutyl paraben and provides strong evidence of an estrogenic mode of action of isobutyl paraben.

REACH registration dossier

The REACH dossier did not include data on reproductive toxicity studies or other *in vivo* or *in vitro* data. Acute toxicity and skin sensitisation and irritation were evaluated based on QSAR prediction using the Danish QSAR database. Genetic toxicity was assessed based on an *in vitro* study. No other information on toxicity testing is available in the REACH dossier.

4.10.3.4 Summary of the plausible link between adverse effects and endocrine mode of action

Several *in vitro* and *in vivo* studies were performed with focus on the endocrine mode of action of isobutyl paraben (Table 1). Most of the *in vitro* studies investigated the estrogenic mode of action of isobutyl paraben and showed estrogenic activity at different levels. The paraben has been shown to bind ERα and ERβ, to upregulate oestrogen regulated genes and proteins and to induce oestrogen regulated proliferation of cells (Kim *et al.* 2012; Kim *et al.* 2011; Vo *et al.* 2010; Dabre *et al.* 2002; Okubo *et al.* 2001). *In vivo* studies confirmed the estrogenic mode of action of isobutyl paraben in uterotrophic assays with s.c. exposure (Koda *et al.* 2005; Dabre *et al.* 2002). At the hormonal level, there are some discrepancies between the studies. Yang *et al.* (2016) showed decreased 17β-estradiol in adult male pups in a developmental study (Yang *et al.* 2016). In contrast, no effects on serum estradiol levels were seen in rat studies with adult exposure in males or females (Kim *et al.* 2015), peripubertal exposure in females (Vo *et al.* 2010) or developmental exposure in females (Kawaguchi *et al.* 2009b). Taken together, the data provide strong evidence of an estrogenic mode of action of isobutyl paraben.

The *in vivo* data on endocrine-related adverse effects of isobutyl paraben are scarce (Table 2). Two studies showed adverse effects that may be related to the estrogenic mode of action shown *in vitro* and *in vivo*. Histopathological changes in female reproductive organs were observed in a female pubertal assay and may be related to an estrogenic effect of the compound (Vo *et al.* 2010). However, the changes reported in the study are not well-described in an extent that makes it unclear what changes were found in the ovaries and the underlying mechanism of action can not readily be discussed. Another study showed adverse effects on male behaviour after perinatal exposure and according to Kawaguchi et al (2009a) the sex-specific anxiogenic affects observed were comparable to behavioural effects seen for gonadal hormones or compounds that mimic oestrogen (Kawaguchi *et al.* 2009a). Another study on behaviour showed impaired social recognition in female offspring exposed perinatally to isobutyl paraben, but it is not clear if the adverse effects are due to an endocrine mode of action. (Kawaguchi *et al.* 2010). Adverse effects on sperm motility and sperm numbers in male pups (Yang et al. 2016) suggest an endocrine mode of action of isobutyl paraben that may be oestrogenic. Altogether, the published data provide moderate evidence of adverse effects linked to an estrogenic mode of action of isobutyl paraben.

The androgenic activity of isobutyl paraben has been investigated to a lesser extent. An *in vitro* androgen receptor binding assay showed activity of isobutyl paraben (Kim *et al.* 2010). On the other hand isobutyl paraben does not appear to affect testosterone levels in *in vivo* studies. No effects on testosterone levels were found in male offspring at weaning or as adults after developmental exposure **(**Yang *et al.* 2016; Kawaguchi et al. 2009b**)** and no effects on testosterone levels were seen in males in a 28-days repeated dose toxicity study with dermal exposure (Kim *et al.* 2015). Adverse effects on sperm motility and sperm numbers in male pups (Yang *et al.* 2016) suggest an endocrine mode of action of isobutyl paraben that may be anti-androgenic. All in all there is weak evidence of adverse effects linked to an anti-androgenic mode of action of isobutyl paraben.

Few studies investigated effects of isobutyl paraben on thyroid hormones *in vivo.* A single study showed effects on serum T4 at PND41 in a female pubertal assay (Vo *et al.* 2010), whereas other studies showed no effects on TT3, TT4 or T3 levels in a 28-days repeated dose toxicity study with dermal exposure and in young and adult pups from a developmental toxicity study (Kim *et al.* 2015; Kawaguchi et al. 2009b). The role of isobutyl paraben on the thyroid hormones is unclear and the changes in T4 levels found by Vo *et al.* (2010) may be a chance finding or due to a possible litter effect. Thyroid hormones are known to be important for brain development and the long-lasting effects on behaviour reported by Kawaguchi and co-workers (2010 and 2009a) showing impaired social recognition in female offspring (Kawaguchi *et al.* 2010) could be linked to thyroid disruption. The mechanisms underlying the behavioural changes were not investigated and the link between these effects and thyroid disruption is unclear.

In summary, data provide strong/moderate evidence of an estrogenic mode of action of isobutyl paraben. Adverse effects on sperm motility and sperm numbers in male pups and effects on sexual dimorphic behaviour suggest an endocrine mode of action of isobutyl paraben that may be oestrogenic. Thus, there is weak to moderate evidence for a causal link. Altogether, the published data provide moderate evidence of adverse effects linked to an endocrine (estrogenic) mode of action of isobutyl paraben.

In conclusion, isobutyl paraben does meet the WHO definition of an endocrine disruptor with an estrogenic mode of action and with a possible anti-androgenic and thyroid disrupting mode of action.

Table 1. Overview of *in vitro* and *in vivo* endocrine disrupting (ED) mode(s) of action (MoA(s)) of isobutyl paraben.

Reference	**MoA**		**Quality of study**	**Evidence for ED MoA**
	In vitro	*In vivo*		
Yang et al. 2016		Decreased 17β-estradiol in male pups after developmental exposure to isobutyl paraben.	Medium	Weak
Kim et al. 2012	Expression of the ER reporter gene and expression of CaBP-9*k* gene and protein was upregulated in all tested doses of isobutyl paraben. The PR gene was upregulated in all the tested doses of isobutyl paraben but only the highest dose (10 µM) upregulated expression of PR protein in GH3 rat pituitary cells.		Medium	Moderate
Kim et al. 2011	22000 fold lower estrogenic activity of isobutyl paraben compared to 17β-oestradiol in a stably transfected transcriptional activation assay in a hERα-HeLa-9903 cell line.		Medium	Weak
Kim et al. 2010	The AR binding affinity relative to dihydrotestosterone was almost 17000 times lower for isobutyl paraben in an androgen receptor binding assay.		Medium	Weak
Vo et al. 2010	Isobutyl paraben had affinity to ERα and ERβ, but did not appear to have a preference to one of the receptors.	Serum levels of T4 were decreased in the low-dose group but not at higher doses.	Low	Weak-weak
Koda et al. 2005		Increased wet and blotted uterus weight in uterotrophic assay with s.c. exposure in ovariectomised rats (n=6/group)	High	Strong
Dabre et al. 2002	Estrogenic activity was seen on binding to ERα, upregulation of oestrogen regulated genes in MCF-7 cells and on oestrogen regulated proliferation in MCF-7 and ZR-75-1 cell lines.	Increased uterus weight (relative to body weight) in uterotrophic study in immature female rats (n=7/ group) with s.c. exposure.	High	Strong

Reference	MoA		Quality of study	Evidence for ED MoA
	In vitro	*In vivo*		
Okubo et al. 2001	Increased proliferation of MCF-7 cells, which was suppressed by the antioestrogen ICI 182,780 interacting with ER, suggesting an estrogenic mode of action of isobutyl paraben. Gene and protein expression of ERα was decreased and gene expression of PR was increased by isobutyl paraben. Isobutyl paraben had 1000-fold lower affinity to human ERα and ERβ compared to the positive control DES.		High	Strong

ER: estrogen receptor; AR: androgen receptor; T4: tetra-iodothyronine; PR: progesterone receptor; s.c.: subcutaneous; DES: diethylstilbestrol

Table 2. Overview of potential endocrine-related adverse effects of isobutyl paraben.

Reference	Species, n	Adverse effects	Quality of study	Evidence for adverse effects
Yang et al. 2016	Rat, n=3 litters/group	Sperm count and motility was reduced by isobutyl paraben in adult male pups after developmental exposure to isobutyl paraben.	Low - Medium	Weak
Kim et al. 2015	Rat, n=10/group	No effects were seen on hormone levels or organ weights in adult rats exposed dermally for 28 days.	Medium	Weak
Kawaguchi et al. 2010	Rat, n=5-6	Female offspring exposed perinatally to isobutyl paraben showed impaired social recognition compared to controls.	High	Strong
Vo et al. 2010	Rat, n=10/group	At all doses myometrial hyperplasia was seen in uteri and in ovaries a decreased number of corpora lutea, increased number of cystic follicles and thinning of the follicular epithelium was apparently observed histologically.	Low	Weak
Kawaguchi et al. 2009a	Rat, n=8/group	Male offspring spent shorter time in the open arms of the elevated plus maze and showed decreased performance in the passive voidance test after developmental exposure to isobutyl paraben.	High	Strong
Kawaguchi et al. 2009b	Rat, n=7-8 litters	Plasma corticosterone levels in dams were decreased after isobutyl paraben exposure and uterus weight was increased.	High	Weak

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