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# Subacute effects of the brominated flame retardants hexabromocyclododecane and tetrabromobisphenol A on hepatic cytochrome P450 levels in rats

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## Abstract

The brominated flame retardants tetrabromobisphenol A (TBBPA) and hexabromocyclododecane (HBCD) are found in the environment, e.g., in sediments and organisms, in food items, human blood samples and mother's milk. In this study, the effects of both compounds on rat hepatic cytochrome P450 (CYP) levels and activities were investigated. Juvenile/young male and female Wistar rats were treated orally with various doses via the feed (TBBPA) or by gavage (HBCD). After 28 days of treatment the animals were sacrificed and hepatic mRNA and microsomes were isolated. HBCD treatment led to a significant induction of CYP2B1 mRNA, CYP2B1/2B2 protein and 7-pentoxyresorufin *O*-depentylase (PROD) activity suggesting a phenobarbital-type of induction. Furthermore, a significant increase in CYP3A1/3A3 mRNA, CYP3A1 protein, and luciferin benzylether debenzylase (LBD) activity was found, being more pronounced in females than in males. The effect on CYP3A1/3A3 mRNA was significant in female rats at a daily dose of 3.0 mg/kg body weight and above. HBCD exhibited no effects on CYP1A2 mRNA, CYP1A1/1A2 protein, or microsomal 7-ethoxyresorufin *O*-deethylase (EROD) activity suggesting lack of activation of the aryl hydrocarbon receptor. No significant effects on any of the parameters measured were obtained with TBBPA. Our findings suggest that oral exposure to HBCD induces drug-metabolising enzymes in rats probably via the CAR/PXR signalling pathway. Induction of CYPs and co-regulated enzymes of phase II of drug metabolism may affect homeostasis of endogenous substrates including steroid and thyroid hormones.

**Keywords:** Brominated flame retardants; Cytochrome P450; Drug metabolism; Hexabromocyclododecane; Induction; Rat liver; Tetrabromobisphenol A

## 1. Introduction

Brominated flame retardants (BFRs) represent a broad spectrum of organic compounds used in a variety of commercial products such as electronic

equipment or textiles. Their widespread production and use has led to increasing contamination in the environment and in detectable levels in humans, which has raised concern on possible adverse health effects (Eriksson et al., 2001; Birnbaum and Staskal, 2004). The most widely investigated group of BFRs, the polybrominated diphenyl ethers (PBDEs) are found in the environment, in wildlife, human tissues (de Boer et al., 1988; Sjödin et al., 1999), and breast milk (Noren and Meironyte, 2000). Their persistence in the environment together with the observation of adverse effects in experimental animals has supported attempts to replace PBDEs by other compounds with BFR properties. 1,2,5,6,9,10-Hexabromocyclododecane (HBCD) and tetrabromobisphenol A (TBBPA) are two prominent examples for such compounds.

HBCD is an additive flame retardant widely used in a number of polystyrene-based materials including resins and fabrics (Alaee et al., 2003). The world-wide demand for HBCD was reported to be over 16,000 t per year in 2001 (BSEF, 2003). HBCD is a highly lipophilic compound which probably shows some persistence in the environment. Its environmental and toxicological properties are currently assessed by a number of institutions. A draft report on this compound has been published by the Swedish National Chemicals Inspectorate (1999).

In isolated rat brain synaptosomes HBCD, in the low micromolar range, inhibits dopamine uptake (Mariussen and Fonnum, 2003). Thyroxin receptor (TR)-mediated gene expression was enhanced in TR-transfected HeLa cells treated with HBCD (Yamada-Okabe et al., 2005). In juvenile rainbow trout, HBCD increased glutathione reductase activity, and inhibited CYP1A-catalysed 7-ethoxyresorufin *O*-deethylase in the liver (Ronisz et al., 2004).

Tetrabromobisphenol A (TBBPA) is used as a reactive flame retardant in plastic materials and as an intermediate in the manufacturing of other flame retardants. It is either incorporated as covalently bound BFR or as an additive to the polymeric mixture. The latter use may lead to leakage, i.e., partial transfer to the environment, food, etc. TBBPA is found in the environment, in human tissues and in breast milk (Noren and Meironyte, 2000). In adult rats, repeated high dosage of TBBPA, in the range of 250 mg/kg b.w., can exhibit porphyrogenic effects, i.e., affect hepatic heme metabolism (Szymanska et al., 2000). In newborn rats, application of TBBPA led to polycystic lesions of the kidney (Fukuda et al., 2004). In the MCF-7 human breast cancer cell line, TBBPA showed weak estrogenic potency (Samuelsen et al., 2001), and acted as a partial estrogen antagonist in the presence of 17β-estradiol (Kitamura et al., 2005a). In the pituitary cell line GH3 it exhibited thyroid hormone-like effects (Kitamura et al., 2005a), whereas it exhibited anti-thyroid hormone effects in thyroid hormone receptor-transfected Chinese hamster ovary cells (Kitamura et al., 2005b). Furthermore, high-affinity binding of TBBPA to the human thyroid hormone transport protein transthyretin was reported by Meerts et al. (2000).

Some BFRs have been suspected to act as endocrine disruptors and/or to affect the development of the unborn. Induction of drug metabolism may play a role in such effects by changing the body's homeostasis of certain hormones such as steroids and thyroid hormones. In particular, induction of drug-metabolising enzymes via the aryl hydrocarbon receptor (AhR), the pregnane X receptor (PXR) or the constitutive androstane receptor (CAR) can interfere with the homeostasis of thyroid hormones and steroids (Mikamo et al., 2003, Xie et al., 2003 and Maglich et al., 2004). In this study, we treated male and female rats with the two widely used BFRs TBBPA or HBCD, and analysed the effects on the hepatic levels of a number of CYP enzymes. We found no effects with TBBPA whereas HBCD

induced hepatic CYP2B and CYP3A enzymes on the levels of mRNA, protein and catalytic activity in a gender-selective manner.

## 2. Materials and methods

### 2.1. Chemicals

A technical mixture of HBCD, composed of samples from a number of producers was obtained through BSEF. The mixture consisted of 10.3% alpha-, 8.7% beta- and 81.0% gamma-HBCD; no data on impurities were provided by the supplier. TBBPA as a technical mixture, homogeneous with a purity of 99.17% and containing tribromo-BPA and *o*,*p*′-TBBPA as impurities (HPLC analysis) was obtained through BSEF (kindly provided by Dr. Klaus Rothenbacher).

For immunodetection the following antibodies were used—CYP1A1/1A2: anti-rat CYP1A1/2 (Daiichi Pure Chemicals, Tokyo, Japan); CYP2B1/2: anti-rat CYP2B1/2 polyclonal antibody (AB1283, Chemicon, Temecula, CA); CYP3A1: anti-rat CYP3A1 polyclonal antibody (AB1253, Chemicon, Temecula, CA). PCR primers were synthesized by MWG-Biotech (Gelsenkirchen, Germany). All other chemicals were of the highest purity commercially available.

### 2.2. Animals and treatment

Male and female rats of the WU (CPB) strain were bred in the RIVM facilities (Bilthoven, The Netherlands), and included in the experiment at approximately 8 weeks of age. Light/dark regime was 12/12 h, and standard pelleted rat feed without soy (Hope Farms/Arie Blok Diervoeding, Woerden, The Netherlands) and drinking water were supplied ad libitum.

The rats were administered either TBBPA or HBCD over 28 days. TBBPA was mixed into the feed. The approximate doses were calculated from feed average consumption as 0, 30, 100, and 300 mg/kg body weight per day, and for each group 10 animals per sex were included. HCBD was administered daily, dissolved in corn oil, by gavage to five animals per sex per dose groups in a dose range of 0, 0.3, 1, 3, 10, 30, 100, and 200 mg/kg body weight per day. This setup with more dose groups compared to the conventional design with four dose groups allows for a more precise analysis of dose responses and subsequent calculation of a critical effect dose at a defined critical effect level. After the end of exposure, animals were anesthetized with $CO_2$ and sacrificed by exsanguination. Necropsies were performed according to OECD407 guideline, enhanced for endocrine and immunological endpoints (Toyoda et al., 2000 and Andrews et al., 2001). Necropsies of females were targeted at the diestrous stage of the estrous cycle. Livers were removed, immediately frozen in liquid nitrogen, and stored at −80 °C until further processing. Experiments were approved by the RIVM Committee on Animal Experimentation according to Dutch legislation.

### 2.3. Hepatic mRNA analysis

Total RNA was isolated with TRIzol (InVitrogen, Karlsruhe, Germany) according to the manufacturer's instructions, and was dissolved in pure water (LiChrosolv, Merck, Germany). Samples were quantified spectrophotometrically using a Nanodrop analyser (Nanodrop, Wilmington, DE), and diluted to 100 ng/µl. Aliquots of RNA were analysed by agarose/formaldehyde gel electrophoresis to control RNA integrity.

Real-time quantitative RT-PCR was performed using an iCycler iQ Real-Time PCR Detection System and iCycler Software version 2.2 (Bio-Rad, Munich, Germany). RT reaction iScript-Kit (Bio-Rad) was used according to the manufacturer's protocol with 100 ng RNA in a final volume of 20 µl. Samples were then assayed in 25 µl reaction mixture using iQ SYBR Green Supermix (Bio-Rad), 6.25 ng of cDNA per reaction, and primers (MWG Biotech, Gelsenkirchen, Germany) as shown in Table 1. The iCycler was programmed: 95 °C 3 min; 40 × (95 °C 1 min, 59 °C 1 min, 72 °C 1 min); 95 °C 1 min; 55 °C 1 min. A melting curve emerging in a gradient from 55 to 95 °C in increasing steps of 0.5 °C verified the single PCR product. $C_t$-values of the target gene were normalized to the housekeeping gene GAPDH, and treated groups were related to the untreated control according to the equation of Pfaffl (2001).

Table 1.

Primers used for real-time PCR

| Gene | GenBank accession | 5′-Sense-3′ | 3′-Antisense-5′ |
| --- | --- | --- | --- |
| rGAPDH | AF106860 | TGCACCACCAACTGCTTAGC | GGCATGGACTGTGGTCATGAG |
| rCYP1A2 | NM_012541 | CGGTGATTGGCAGAGATCGG | GTCCCTCGTTGTGCTGTGG |
| rCYP2B1 | M37134 | ATGGAGAAGGAGAAGTCGAACC | CTTGAGCATCAGCAGGAAACC |
| rCYP3A1/3 | NM_173144/NM_013105 | CCAGCAGCACACACTTTCCTTTG | GGTGGGAGGTGCCTTATTGG |

### 2.4. Western blotting of CYP enzymes

Vertical SDS gel electrophoresis and semi-dry membrane blotting were carried out with 20 µg protein per lane as described previously (Zeiger et al., 2001). Immuno-reactive bands were visualized with the ECL-Plus detection kit (Amersham, Braunschweig, Germany) using a Lumi-Imager (Roche, Basel, Switzerland).

### 2.5. Hepatic enzyme activity assays

Microsomes were isolated according to the protocol of Melancon et al. (1985), and dissolved in 50 mM sodium phosphate buffer, pH 7.4. EROD and PROD activities were measured in triplicates in multiwells using a Fluoroskan Ascent FL microplate reader (Labsystems, Vantaa, Finland) according to the methods described by Bohnenberger et al. (2001). Total protein was determined according to Kennedy et al. (1995) using the fluorescamin reaction. LBD activity was analysed with 13 µg microsomal protein using the P450-Glo™ CYP3A4 Assay (Promega, Heidelberg, Germany) according to the manufacturer's protocol.

### 2.6. Statistical analysis

Comparison of means between treated groups and the control group were carried out using Dunnett's test for multiple comparisons with a control. Trend analysis was carried out according to Duckworth and Wyatt.

## 3. Results

The brominated flame retardants TBBPA or HBCD (Fig. 1) were given to juvenile/young WU (CPB) rats of both sexes. TBBPA was applied in the feed and the average doses were estimated from feed consumption based on daily weighing of the feed. HBCD was given daily by gavage. After 28 days, animals were sacrificed and livers were removed for further analysis.

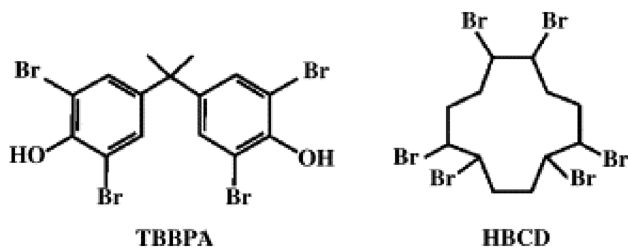


*Fig. 1. Chemical structure of tetrabromobisphenol A (TBBPA) and hexabromocyclododecane (HBCD).*

In liver microsomes, no effects of TBBPA were observed on CYP-catalysed EROD, PROD or LBD activities (Table 2A). Analysis of specific mRNAs by real-time PCR did not reveal any significant effects of TBBPA on the transcripts of CYP1A2, CYP2B1, and CYP3A1/3A3 (Fig. 2). A similar picture was obtained for the corresponding proteins analysed by Western blotting (Fig. 3). An apparent trend for increasing levels of CYP3A1 in TBBPA-treated females was not confirmed by statistical analysis.

Table 2A.

CYP activities in liver microsomes isolated from TBBPA-treated rats

| **Treatment (dose in mg/kg body weight per day)** | **Male** | | | **Female** | | |
|---|---|---|---|---|---|---|
| | **EROD (pmol/ mg protein/min)** | **PROD (pmol/ mg protein/min)** | **LBD (fluorescence units/ mg protein)** | **EROD (pmol/ mg protein/min)** | **PROD (pmol/ mg protein/min)** | **LBD (fluorescence units/mg protein)** |
| Control (DMSO) | 22 ± 14 | 27 ± 21 | 1.0 ± 0.3 | 32 ± 15 | 42 ± 21 | 1.0 ± 0.4 |
| TBBPA 30 | 21 ± 10 | 22 ± 18 | 1.0 ± 0.4 | 23 ± 9 | 24 ± 16 | 0.6 ± 0.4 |
| TBBPA 100 | 24 ± 14 | 33 ± 21 | 1.9 ± 1.4 | 25 ± 16 | 37 ± 23 | 0.6 ± 0.2 |
| TBBPA 300 | 23 ± 8 | 28 ± 19 | 1.1 ± 0.6 | 23 ± 5 | 28 ± 20 | 0.8 ± 0.3 |

Data represent means ± S.D. from three independent experiments.

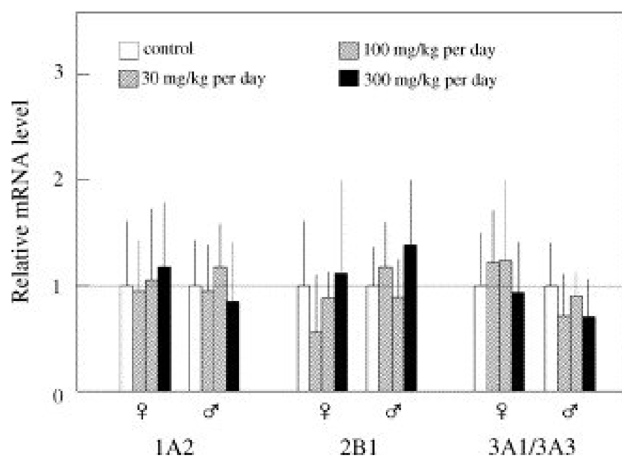


*Fig. 2. Effects of various doses of tetrabromobisphenol A (TBBPA) given orally via the feed over 28 days on hepatic mRNAs of various cytochromes P450 (CYP) in male and female rats. Average doses were estimated from feed consumption data. *Significantly different from controls ($p \leq 0.05$). Data represent means ± S.D. from three independent experiments.*

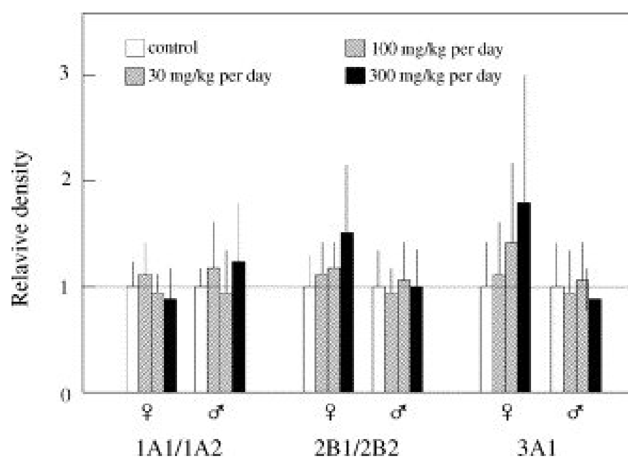


*Fig. 3. Effects of various doses of tetrabromobisphenol A (TBBPA) given orally via the feed over 28 days on hepatic levels of various cytochrome P450 (CYP) enzymes in male and female rats. Average doses were estimated from feed consumption data. *Significantly different from controls ($p \leq 0.05$). Data represent means ± S.D. from three independent experiments.*

The second flame retardant investigated was HBCD. HBCD led to a significant induction of PROD activity in male rats at 10 and 100 mg/kg body weight per day, and of LBD activity in female rats at 10, 30 and 200 mg/kg body weight per day (Table 2B). At 30 mg/kg body weight per day PROD activity in males seemed to be slightly, but not significantly increased. LBD activity in microsomes from females also seemed to be increased at 100 mg/kg body weight per day. However, the effect was not statistically significant. No trends were observed for any of the other activities including EROD activity.

Table 2B.

CYP activities in liver microsomes isolated from HBCD-treated rats

| Treatment (dose in mg/kg body weight per day) | Male | | | Female | | |
|---|---|---|---|---|---|---|
| | EROD (pmol/mg protein/min) | PROD (pmol/mg protein/min) | LBD (fluorescence units/mg protein) | EROD (pmol/mg protein/min) | PROD (pmol/mg protein/min) | LBD (fluorescence units/mg protein) |
| Control (DMSO) | 25 ± 7 | 30 ± 6 | 1.0 ± 0.4 | 28 ± 7 | 31 ± 11 | 1.0 ± 1.0 |
| HBCD 0.3 | 27 ± 5 | 35 ± 10 | 1.3 ± 0.5 | 29 ± 7 | 30 ± 2 | 1.2 ± 0.8 |
| HBCD 1.0 | 28 ± 3 | 39 ± 7 | 1.1 ± 0.3 | 30 ± 6 | 37 ± 7 | 1.8 ± 1.0 |
| HBCD 3.0 | 24 ± 4 | 37 ± 5 | 0.4 ± 0.4 | 25 ± 5 | 28 ± 4 | 1.7 ± 1.1 |
| HBCD 10 | 23 ± 2 | 45 ± 6* | 0.9 ± 0.3 | 26 ± 5 | 35 ± 7 | 3.8 ± 2.5* |
| HBCD 30 | 25 ± 5 | 41 ± 6 | 0.6 ± 0.6 | 27 ± 6 | 32 ± 4 | 5.0 ± 3.9* |
| HBCD 100 | 31 ± 7 | 51 ± 7* | 1.7 ± 1.2 | 26 ± 5 | 35 ± 7 | 4.1 ± 2.3 |
| HBCD 200 | 27 ± 6 | 35 ± 8 | 1.4 ± 0.8 | 26 ± 4 | 26 ± 5 | 10.2 ± 5.2* |

Data represent means ± S.D. from three independent experiments.
* Significantly different from controls ($p \leq 0.05$).

In contrast to TBBPA, HBCD was an effective inducer of CYP2B1 mRNA in females, and a weaker but significant inducer in males (Fig. 4). The standard deviation shown in Fig. 4 for the effect on CYP2B1 mRNA in females at the highest dose reflects the high variability of this response on the transcript level. Nevertheless, pronounced induction was observed in all animals of the corresponding group, while the degree of induction was variable.

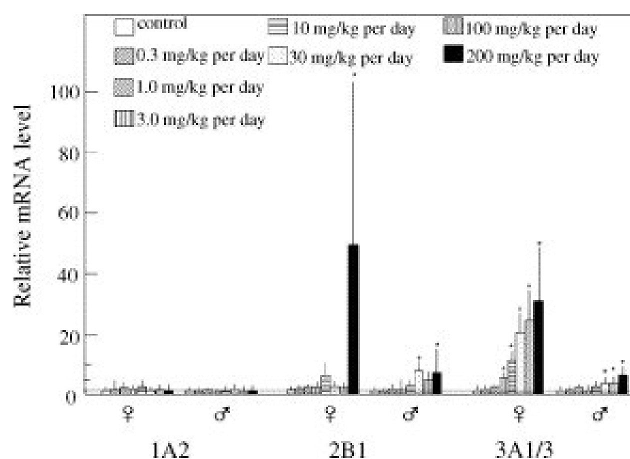


*Fig. 4. Effects of various doses of hexabromocyclododecane (HBCD) given orally by gavage over 28 days on hepatic mRNAs of various cytochromes P450 (CYP) in male and female rats. *Significantly different from controls (p ≤ 0.05). Data represent means ± S.D. from three independent experiments.*

Induction of CYP3A1/3A3 mRNAs was also much more pronounced in females than in male animals and reached significance at a dose level of 3 mg/kg body weight per day. In males significant but much lower induction was observed at 30 mg/kg body weight per day and above. Analysis of AhR-regulated CYP1A2 mRNA did not reveal any significant effects.

Fig. 5 shows the results of Western blot analysis of CYP enzymes in liver microsomes from HBCD-treated rats. It was found that HBCD led to a dose-dependent significant induction of CYP2B1/2B2 in females and a trend for induction in males. Furthermore, CYP3A1 protein was induced in females at a daily dose of 3 mg/kg body weight and above, in males at 30 mg/kg body weight and above, the effect in females being much stronger than in males. No effects on the levels of CYP1A1/1A2 were observed.

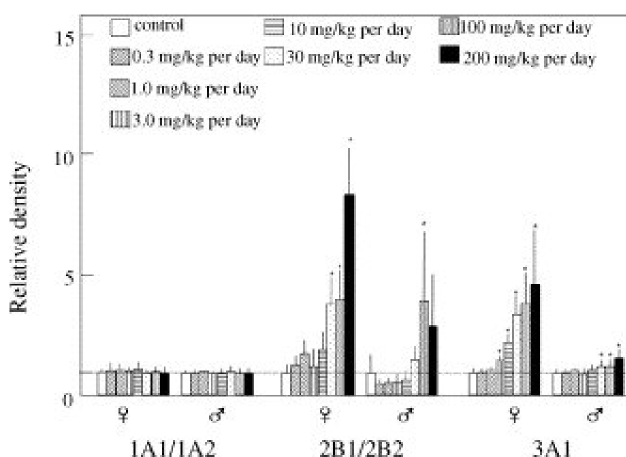


*Fig. 5. Effects of various doses of hexabromocyclododecane (HBCD) given orally by gavage over 28 days on hepatic levels of various cytochrome P450 (CYP) enzymes in male and female rats. *Significantly different from controls ($p \leq 0.05$). Data represent means ± S.D. from three independent experiments.*

## 4. Discussion

The brominated flame retardants TBBPA and HBCD are found in the environment including sediments and soils, lower and higher organisms (de Boer et al., 1988; Sjödin et al., 1999). Furthermore, their production and use resulted in widespread exposure of humans via the food leading to detectable levels in blood and mother's milk (Noren and Meironyte, 2000). In comparison to the polybrominated diphenylethers, another class of brominated flame retardants, very few information is available on toxic effects of these compounds in mammalians. Reports on interaction of TBBPA with the thyroid hormone receptor led to concerns about possible interactions of this compound with thyroid hormone homeostasis.

A hallmark of PXR activation is induction of CYP3A1/3 (Kliewer et al., 2002). Other signalling pathways regulating the levels of CYPs include CYP2B1/2B2 induction mediated by CAR, and CYP1A1/1A2 induction mediated by the AhR.

Our data suggest that TBBPA does not affect any of these pathways. This conclusion is based on the lack of significant effects of the compound on the

mRNA levels, protein levels, and catalytic activities of the indicator enzymes investigated.

In contrast, HBCD was found to act as an inducer of CYP2B1/2B2 mRNA in rats of both sexes. In contrast to males, HBCD acted as a highly effective inducer in females at the highest dose level only. However, no effects on PROD activity, indicative for induction of CYP2B1/2B2 function were detected in females. It is noteworthy that all female rats in this dose group showed markedly increased CYP2B1/2B2 mRNA levels, the high standard deviation being due to the fact that the extend of induction was highly variable. A possible explanation for the discrepancy between the effects on CYP2B1/2B2 mRNA and the lack of PROD induction may be that high levels of HBCD sufficient to induce CYP2B1/2B2 mRNA may inhibit PROD activity. Furthermore, a dose-dependent increase in immuno-detectable CYP2B1 protein in females confirms the notion that HBCD is a 'phenobarbital'-type inducer of drug metabolism in rats.

A consistent dose–response was found for induction of PXR-regulated CYP3A1/3A3 mRNA. Here, the lowest effective dose was 3.0 mg/kg body weight per day in females. In females, the inducing effect was much more pronounced than in males which is in agreement with the female-predominant expression of CYP3A1 in rats (Thangavel et al., 2004).

Our findings indicate that CYP3A1/3A3 induction is a major effect of HBCD and seems to be among the most sensitive effects of these compounds in higher animals reported so far. In humans, PXR-regulated CYP3A enzymes, most notably CYP3A4, and CAR-regulated CYP2B enzymes, most notably human CYP2B6, metabolize a broad spectrum of drugs (Maurel, 1996 and Ekins and Wrighton, 1999). In rats both CYP3A1 and CYP2B1 catalyse the hydroxylation of testosterone in the liver (Elias and Gwinup, 1980). As a consequence, phenobarbital-type induction may increase hepatic androgen metabolism, e.g. during male development, and could result in androgenic deficiency, reproductive failure and demasculinization.

Since the induction is likely to be mediated via the rat PXR, it appears plausible to anticipate that other PXR-regulated genes are also induced by HBCD. In fact, PXR and CAR also regulate the expression of certain phase II enzymes including UGT 1A1, the major thyroxin-conjugating UGT (Mackenzie et al., 2003). One mechanism by which xenobiotics can affect thyroid hormone levels in blood is via induction of UGTs which conjugate thyroxin (T4) and make it better excretable by the kidneys. It has been reported by Wong et al. (2005) that PXR-driven induction of UGTs together with the biliary conjugate export pump Mrp2 in rats can lead to a massive decrease in blood thyroxin levels. A similar mechanism has been suggested for CAR-driven expression of conjugating enzymes (Wong et al., 2005). Decreased T4 may affect the development of the unborn in particular of the central nervous system and its functions (Morreale de Escobar et al., 2004). Furthermore, a compensatory rise in thyroid stimulating hormone (TSH) aimed at counterbalancing hypothyroidism may lead to a sustained proliferative stimulus on the thyroid and may thus be related to the occurrence of thyroid tumors (Vansell et al., 2004).

Thus alterations of levels and activities of drug-metabolising enzymes such as CYP3A1/CYP3A3 and UGTs may affect the homeostasis of steroid and thyroid hormones, e.g., in the maternal organism and may thus affect the development of the offspring. Induction of drug metabolism with consequences for the homeostasis of thyroid hormones and the development of the unborn were reported for brominated diphenyl ethers (Zhou et al., 2002). However, hepatic

mechanisms of reduction of circulating thyroid hormones found in rats do not necessarily cause similar effects in humans (Ohnhaus et al., 1981).

Since activation of PXR has been reported for a broad spectrum of chemicals including halogenated pesticides and related compounds (Lemaire et al., 2004), our findings with HBCD are not surprising. However, profound differences exist between human and rat PXR with respect to activation by various agonists (Xie et al., 2002). Likewise, rifampicin is considered as a prototype agonist of hPXR, dexamethasone of rPXR. Since such differences may also exist for HBCD, our findings do not allow a direct extrapolation from rats to humans. Furthermore, CAR and PXR can regulate the same genes as has been shown by Xie et al. (2002) for the human UGT locus demonstrating the need for further investigations on possible distinct effects of HBCD on both transcription factors.

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