ENDOCRINE DISRUPTING EFFECTS OF SELECTED BROMINATED FLAME RETARDANTS IN RATS

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Introduction

Brominated flame retardants (BFRs) are compounds used to prevent accidental fire in consumer products due to overheating. These life-saving agents are covalently bound to or mixed in inflammable materials such as polysterenes, other construction materials, printed wiring boards, plastic casings, upholstery and textiles. BFRs are widely used and therefore produced in large volumes. Most BFRs are persistant pollutants and have been recovered from many environmental compartments as well as from human blood and breast milk. The EU-sponsored FIRE project aims at integrated risk assessment of these BFRs, with a focus on endocrine disruptive effects. A major theme in this project is hazard identification for humans, by studying the effects in a rodent model. Four brominated flame retardants (BFRs) were selected on the basis of production volume, exposure data, QSAR and in vitro prescreening results[1]. These BFRs were: tetrabromobisphenol-A (TBBPA), hexabromocyclododecane (HBCD), and the penta- and decabrominated diphenylethers (pBDE and dBDE).

Materials and Methods

Wistar rats were purchased from Harlan (Horst, NL), or bred at the RIVM facilities. All four BFRs were industrial mixtures obtained through BSEF (dr. Klaus Rothenbacher) or provided by Great Lakes Chemical Corperation (pBDE, Dr. D. Sanders); pBDE was purified to remove dioxins, dibenzofurans, and any other coplanar molecules (dr. Åke Bergman). TBBPA was mixed in the diet, HBCD and pBDE were dissolved in corn oil, then given by daily gavage (HBCD and pBDE 28d studies) or by mixing in the diet (HBCD reproduction study), dBDE was administered by gavage in an emulsion. The compounds were tested in 28d repeated oral dose toxicity studies (OECD407 protocol), TBBPA and HBCD also in one-generation reproduction studies (OECD415). The protocols were enhanced for endocrine and immunological endpoints[2]. For precise assessment of dose-response relationships, the animals were distributed among eight dose groups (including control). This setup enables benchmark dose (BMDL) calculations[3], i.e. the 5% lower confidence bound of the critical effect dose (CED) at a critical effect size (CES), which was defined at a 10 % change for most parameters. Exposure started after at least one week of acclimatization in animals 8-12 w of age. Dose ranges were 3-3000 mg per kg body weight for TBBPA, 0.3-200 mg/kg for HBCD, 0.27-200 mg/kg for pBDE, and 1.87-60 mg/kg for dBDE.
Materials were collected during necropsy of all animals in the 28d studies and the same number of F1 animals in the reproduction studies, i.e. five animals per sex per dose group for assessments of cauda epididymis sperm, of (immune) cell subpopulations and/or NK activity in whole blood, bone marrow, and/or part of the spleen[4], and of weight and histopathology of a conventional set of organs. TSH, FSH, LH and prolactin in the adenohypophysis were analyzed by immunohistochemistry when indicated by other effects. Left femur and tibia were prepared for physical bone analyses, which are reported seperately[5]. Various organ and tissue samples were stored under appropriate conditions for further analysis, including routine plasma clinical chemistry and hormone analysis (mainly thyroid hormones[6]). Effects on drug metabolism were assesesed by analysing hepatic P450 at the level of mRNA, protein, and enzyme activity (reported seperately[7,8]). Effects on the production of sex steroid hormones were assessed by analysing the activity of CYP19 (aromatase; key enzyme for estrogen synthesis) by a radioactive assay[9], in ovaries, and by measuring DHEA, which is the product of CYP17 (17-hydroxylase/17,20lyase; key enzyme of androgen synthesis) with a RIA (Immunotech, Bechman Coulter, Mijdrecht NL) in adrenals. Control positive and negatives were included in both assays. Additional parameters in the reproduction studies included neonatal status and viability, and developmental endpoints in pups. Young

adult F1animals were also used for immunocompetence[10] and neurobehavioural (reported seperately[11]) assessments. Internal dosing was verified by compound analysis in liver samples by LC-MS/MS after gradient separation in a HPLC column. Experiments were approved by the institutional Committee on Animal Experimentation, according to Dutch legislation.

Results and Discussion

All four tested BFRs induced multiple effects, each compound with a specific pattern. As a common finding, there were sensitive effects in the thyroid hormone domain. TBBPA and dBDE induced increased TT3, both only in females (Table 1), which was associated with decreased TT4 (females and males) in the case of TBBPA. HBCD induced increased thyroid gland weight, also with associated decrease of TT4 (both only in females), and decreased TT4 was also observed with pBDE (bot sexes). A possible explanation for the decreased TT4 is that the BFRs evoke a detoxification response in the liver, which concommittantly affects T4. Indeed, this was supported by the increased liver weight observed to variable extents with TBBPA, HBCD, and pBDE, which was associated with histopathological changes in the HBCD study (hepatocellular basophilia, suggestive of induction of endoplasmatic reticulum), and in the pBDE study (centrilobular hypertrophy). In the HBCD study, this was further supported by induction of P450 CYP2B and CYP3A mRNA, protein, and enzyme activity[7] and induction of T4-UDP glucuronyl transferase. These enzyme systems are known to be co-expressed and to co-operate, to facilitate multistep drug metabolism[12]. A similar stimulation of drug metabolism was observed with pBDE[8]. P450 induction was not observed in the 28d study with TBBPA (not analysed in the reproduction study)[7]. One of several other mechanisms which can induce decreased TT4 is increased deiodination[13], which was not analyzed, but could be consistent with the increased TT3 levels in the TBBPA and dBDE studies. Increased metabolisation of T4 could be enhanced by increased availability of T4 due to competition of the BFR on the plasma TH carrier, transthyretin (TTR). In vitro studies indicated that this is a potential mechanism with TBBPA, but unlikely in the case of dBDE[1,14]. Furthermore, the E2-dependent lower T4 binding of TTR[15] may facilitate competitive displacement of TH by TBBPA, which supports the gender specificity of increased TT3.

Follow-up events of effects on TH concentrations include feedback on the TH axis, as was most evident in the HBCD 28d study, which showed increased pituitary weight and increased immunostaining of TSH in the pituitary, increased thyroid weight and thyroid follicle cell activation (all in f only). Similarly, there was an increased pituitary weight (m only) and slight activation of the thyroid gland (f only) in the TBBPA reproduction study. Remarkably, no such changes were observed in the study with pBDE. In view of functionality of TH, some changes in clinical chemistry parameters could be considered as secondary effects of a disrupted TH balance. In this way, the increased plasma cholesterol observed in the studies with HBCD and pBDE may reflect the T4 hypothyroidy, which results in an impaired balance between degradation and synthesis of lipids[16,17]. In contrast, in males, a TH mediated stimulating effect of HBCD, known from in vitro studies[1] could then dominate in males, thus explaining the decreased cholesterol. Similarly, the increased plasma protein levels, observed particularly with pBDE (at high dose levels also with HBCD, not shown), could also result from an impaired balance between protein synthesis and degradation, which is a known effect of hypothyroidy[18]. Although such clinical chemical endpoints are multi-interpretable, they may be valuable in the overall context to estimate functional implications of BFR-induced effects. This is certainly true for developmental effects in the neurophysiological functions, reported elsewhere, which were observed in the TBBPA and HBCD reproduction studies, and are for a large part also considered as secondary to TH changes[11].

Gender-dependent differences, which were apparent for most BFR-induced effects, were largely consistent with gender-specific effects on TH plasma levels. Notably, in the case of HBCD, effects associated with the TH hormone domain, including activation of the liver and hepatic metabolizing enzymes, T4 hypothyroidy, and activation of pituitary and thyroid glands, were consistently restricted to females. These gender differences may be related to gender-specific differences in uptake and/or metabolism of the BFRs, as illustrated by the higher internal concentrations of HBCD in the liver in females.

The BFRs also induced varying effects on the immune system, i.e. on thymus weight, spleen cellularity and blood monocyte counts. Although some of these effects occurred at low dose (Table 1), they were not associated with functional effects (Natural Killer activity and immunization efficacy test), and their hazard therefore remains unknown. The effects on immune parameters can be understood as a direct interaction of the BFR with the immune system, but indirect actions can also be involved. In this respect, TH could be a mediator of effects, because TH are known to interact with the immune system[19].

Table 1 – Summary of sensitive effects* in rats of brominated flame retardants

		males		*females*	
	parameter	*BMDL*	*max response*	*BMDL*	*max response*
TBBPA	**pituitary weight**	**0.6**	+46		
	plasma total T3			1.9	+27
	plasma total T4	17	-60	28	-56
HBCD	**thyroid weight**			**1.6**	+61
	T4-UGT			4.1	+121
	pituitary weight			30	>+41
	TT4			56	>-24
	total cells per spleen	1.7	-28	nd	
	CD4 (Th)	0.3	-33	nd	
	CD161a (NK)	6.3	-41	nd	
	liver weight			23	+36
	cholesterol	66	<-21	7	+20
	alkaline phosphatase			19	>- 45
	glucose	57	<-20	71	>-17
pBDE	**TT4**	**1.1**	-88	1.8	-89
	body growth w 1-4	3.8	>-53		
	liver weight	18	>+97	17	+61
	epididymis weight	28	>-14		
	seminal vesicle weight	51	>-18		
	prostate weight	43	>-30		
	thymus weight	110	>-19		
	cholesterol	8.5	>+105	12	>+144
	total protein			12	>+12
	alanin aminotransferase	16	>+151		
	glucose	67	>-41		
	urea	30	>+35	22	>+34
	kidney weight	109	>+12	85	>+14
dBDE	**TT3**			**33**	>+21
	thymus weight			43	>-17
	brain weight			69	>-5

BMDL is benchmark dose (mg/kg bw) determined at the lower confidence bound of the 95% confidence interval of the critical effect dose (CED). CEDs were based on a standard effect size of 10%, except for immune parameters and liver weight (20%). Results are from 28d studies, except with TBBPA, which shows results in young adult F1 animals of the reproduction study at final necropsy. Maximal response is at plateau of the dose response curve (or at max. dose with > or < signs), and expressed as the percentage relative to background level (mg / kg bw). *Only effects in the lower dose range are reported here. Sensitive parameters that are potential determinants for risk assessment are highlighted. nd, not determined.

In the TBBPA reproduction study, there was a set of effects related to growth, development and reproduction with a suggested nonmonotonic (U-shaped) dose-response, which therefore were not suitable for benchmark analysis. These effects included decreased mortality during lactation, decreased ano-vaginal distance pnd7, increased weight of uterus/ovaries pnd21, weight change of testis and uterus, increased endometrium thickness, and increased CYP19 in the ovary, the latter four at terminal necropsy of F1. In view of the deviating dose-response pattern, these effects were probably unrelated to the effect on the TH axis in this study.

Further effects on steroidogenesis included induction of CYP19 in the ovary and of CYP17 in the adrenals in the mid-dose groups of the HBCD/28d study, although without statistical significance. In the pBDE study, induction of CYP17 was detected in female groups (maximal induction in the submaximal dose group; no effect observed in the top dose group). Dystrophic changes which were observed in the zona reticularis of the adrenals were not obviously correlated to CYP17 induction.
In conclusion, most important effects of the tested BFRs were related to the TH domain, either because of involvement of TH metabolism, or because they were secondary to changes in TH levels. Other, TH independent, effects were related to the sex steroid domain and to the immune system. The most sensitive effects, i.e. with the lowest BMDLs (highlighted in Table 1) will be used for risk assessment of these BFRs.

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