Polybrominated Diphenyl Ethers

Aspects of the mechanism of action

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Gebromeerde Diphenyl Ethers

Enkele aspecten van het werkingsmechanisme

(met een samenvatting in het Nederlands)

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Annelieke Katrien Peters
geboren op 30 juni 1978, te Eindhoven

promotor Prof. Dr. M. Van den Berg
Institute for Risk Assessment Sciences (IRAS)
Universiteit Utrecht

co-promotor Dr. J.T. Sanderson
Institut National de la Recherche Scientifique-Institut Armand-Frappier (INRS-IAF), University of British Columbia, Canada

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Peters, Annelieke K.

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We are surrounded by flame retardants, both in our home as well as in our work environment. These chemicals are added to a variety of household and office products to meet fire safety standards.
Unfortunately, some of the chemicals used as flame retardants do not remain in the polymers they are added to; as a consequence these chemicals can leach out of these products into the environment.

One group of flame retardant chemicals is the group of brominated flame retardants. These can be found worldwide, persist in our environment and bioaccumulate in food chains. These properties cause concern and were the basis for the author to study some aspects of the mechanism of action of one class of brominated flame retardants, the polybrominated diphenyl ethers (PBDEs).

AhR	aryl hydrocarbon receptor
ARNT	AhR nuclear translocator
BDE	brominated diphenyl ether
bp	base pairs
BFR	brominated flame retardants
CAR	constitutive androstane receptor
CYP1A1	Cytochrome P450 1A1
CYP1A2	Cytochrome P450 1A2
CYP1B1	Cytochrome P450 1B1
CYP3A4	Cytochrome P450 3A4
deca-BDE	PBDE with ten bromine substitutions
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DRE	dioxin responsive element
EC_{25}	the concentration that is needed to induce 25% of the maximum response
EC_{50}	the concentration that is needed to induce 50% of the maximum response
$EC_{5\ TCDD}$	the concentration that is needed to induce a response relative to the response of TCDD (5nM)
EGFP	enhanced green fluorescent protein
EROD	7-ethoxyresorufin-O-deethylase
FCS	fetal calf serum
FBS	fetal bovine serum
Gal4	yeast protein that is a transcriptional activator and binds to DNA
H4IIE	rat hepatoma cell line
HAH	halogenated aromatic hydrocarbons
HBCD	hexabromocyclododecane
HepG2	human hepatocellular liver carcinoma cell line
H1G1.1c3	stably transfected mouse hepatoma cell line containing an AhR-responsive EGFP reporter gene
H4G1.1c2	stably transfected rat hepatoma cell line containing an AhR-responsive EGFP reporter gene
IC_{50}	the concentration that is needed to inhibit half of the maximal response
IUPAC	International Union of Pure and Applied Chemistry
LD_{50}	the concentration that proved lethal for 50%
LOEL	lowest observed effect level
Luc	luciferase
MCF-7	human breast adenocarcinoma cell line
MO-PCB	mono-ortho polychlorinated biphenyl
mRNA	messenger ribonucleic acid
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NOEL	no observed effect level
Octa-BDE	PBDE with eight bromine substitutions
OECD	Organization for Economic Co-operation and Development
PAH	polynuclear aromatic hydrocarbons
PB	phenobarbital
PBB	polybrominated biphenyl
PBDD	polybrominated dibenzo-p-dioxins
PBDE	polybrominated diphenyl ether
PBDF	polybrominated dibenzofurans
PBS	phosphate buffered saline
PCB	polychlorinated biphenyl
PCR	polymerase chain reaction
PHAH	planar halogenated aromatic hydrocarbons

Penta-BDE	PBDE with five bromine substitutions
POP	persistent organic pollutant
PXR	pregnane X receptor
qPCR	quantitative polymerase chain reaction
REP	Relative potency value
RNA	ribonucleic acid
RT	reverse transcriptase
RoHs	Restriction of Hazardous Substances Directive
ROS	restriction of substances
RXR	9-*cis* retinoic acid receptor
SAR	structure activity relationships
SD	standard deviation
SEM	standard error of the mean
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TBBPA	tetrabromobisphenol A
TEF	toxic equivalency factor
TEQ	toxic equivalency
XRE	xenobiotic response element

CHAPTER ONE

General Introduction

Introduction

During the last decades, a large variety of synthetic chemicals has entered the environment due to their use in agricultural, industrial, or household applications. The entry of these chemicals into the environment can be intentional, such as herbicides and pesticides, or unintentional, such as polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs) and polybrominated diphenyl ethers (PBDEs). These groups of compounds have entered the environment as by-products of the industrial manufacturing or combustion process, leakage, or leaching during the product's lifetime or after disposal.

1 Polybrominated diphenyl ethers

PBDEs are a class of brominated flame retardants (BFRs) used as additives in various consumer products such as plastic materials, paints, and textile fabrics since the 1970s at concentrations of 5-30% to reduce and retard the development of a fire (see Box 1). Demands for flame retardant chemicals have grown strongly as product safety standards have tightened more during the last decades. PBDE molecules have an ether bridge between the two phenyl rings, resulting in the inability to adopt a planar configuration. The substitution pattern of the bromines is like the chlorination pattern of PCBs with 209 possible congeners, from mono- to deca-BDE hence, the numbering of the PBDE congeners is adapted from the International Union of Pure and Applied Chemistry (IUPAC) system and is the same as the numbering of PCB congeners [5].
There are three commercial mixtures of PBDEs in use, differing in their degree of bromination: Penta-BDE; Octa-BDE; and Deca-BDE (see Box 1, Table 1). The worldwide estimated demand for PBDEs in 2001 was estimated to be almost 70.000 tonnes, with Deca-BDE accounting for almost 80% of the total market (Source: Bromine Science and Environmental Forum, Brussels, Belgium).

Although PBDEs have a clear benefit in the way that they reduce the chances of ignition and burning of materials, they have the ability to leach out of the polymers in which they are present.
As a result of the large scale production and use, PBDEs became ubiquitous environmental contaminants with increased levels in soil and sludge samples [16,17], fish and wildlife [18-22], and in human blood, adipose tissue [27], and breast milk samples [31,32].

Structural similarities of PBDEs with other polyhalogenated aromatic hydrocarbons that bind to the aryl hydrocarbon receptor (AhR), such as some polychlorinated biphenyls (PCBs) raised concern about the possible binding of PBDEs to the AhR, leading to AhR-mediated toxic and biological effects.

Chemical name	General information	Used in
Penta-BDE (cas no 32534-81-9)	$C_{12}H_5Br_5O$ log Kow 6.5-7.0	polyurethane foams in furniture and some building materials
Octa-BDE (cas no 32536-52-0)	$C_{12}H_2Br_8O$ log Kow 8.4-8.9	housings for television sets, computer casings and other electronics
Deca-BDE (cas no 1163-19-5)	$C_{12}Br_{10}O$ log Kow 10	housings for television sets, computer casings and various plastics, paints, wire and cable insulation, coatings and adhesive systems

Table 1; Chemical properties of the commercial PBDE mixtures, Penta-BDE, Octa-BDE, and Deca-BDE [1,33-36]

2 Kinetics and metabolism of PBDEs

2.1 Abiotic samples

PBDEs have been detected in air particulates all over the world [37-39], BDE-47 and -99 were the predominant reported congeners, though BDE-209 was often not included. The highest reported concentration was 52 pg/m^3 near the city of Chicago [37]. The levels detected in sewage sludge range from 13-270 ng/g dry weight with the highest levels detected in the vicinity of industry sites, indicating that industry was the most likely source. In sediments of rivers in Europe, it was apparent that concentrations (ΣPBDEs) varied almost 150 times (14.1-2250 ng/g) between samples taken upstream and downstream of suspected sources [16,17,19,40]. In sediment, deca-BDE is often the predominant detected congener.

There was a peak in PBDE concentrations in the environmental levels in the mid 80's and after this levels either decreased or remained the same [26].

It has been postulated that the Deca-BDE levels in the environment were influenced by microbial degradation in anaerobic sediments and degradation of the compound under the influence of UV-light and sunlight to lower brominated congeners [41-43].

2.2 *In vivo* experiments

Most data concerning the absorption of PBDEs is available for BDE-47 and BDE-99 and results seem to be directly related to the number of bromines in the molecule; more bromine atoms decrease the bioavailability. In most toxicological studies, PBDEs were administered orally to rodents with the main storage sites being lipid rich adipose tissues such as the liver [44,45].

Limited data is available about the half-lives of individual PBDEs; they range from 30 to 90 days for the tetra- to hexa-substituted congeners in rodents. LD_{50} values for PBDEs mixtures are >2000 mg/kg bodyweight with a NOEL of 1 mg/kg bw/day [45,46].

Intestinal absorption of the deca-BDE is limited in rodents; more than 90% of an orally given dose is normally excreted in the faeces, but there is a strong influence by the nature of the carrier [47,48]. These results indicate that deca-BDE uptake is not efficient, it has a short half-live and consequently has very low bioaccumulating potential [19,32,45]. For the lower brominated tetra- and penta-BDEs more than 80% of an oral dose was absorbed and stored in the most lipid rich tissues [44,45]. With respect to toxicokinetics there are clear intra-species differences; for example the urinary excretion of BDE-47 is a minor excretion route in rats, but as important as faecal excretion in mice [45]. Dermal uptake of deca-BDE seems to be low, estimated at 4.5%, while for BDE-47 ~62% of a dose was adsorbed when administered dermally [48]. Studies in pregnant mice showed low fetal uptake, though breast milk uptake was substantial and 30-40% of the administered single dose was found in the suckling offspring [49].

In fish and shellfish, uptake rates were approximately ten times higher for the lower brominated BDE-47 and -99 than for higher brominated congeners such as BDE-153 [50,51]. In fish, PBDEs accumulated in the liver, gall bladder, kidneys, brain, chorion of the eye and adipose tissue along the spinal column [50].

In both *in vivo* and *in vitro* laboratory studies PBDEs have been shown to interfere with the thyroid hormone systems [52-55], resulting in decreased thyroid hormone thyroxine levels and hyperplasia of the thyroid in rats and mice. With respect to effects reported on the thyroid hormone homeostasis, it has been suggested based on *in vitro* studies that these are caused via CYP-P450 mediated metabolic conversion of PBDEs into hydroxylated metabolites [56]. However, in perinatally exposed mice, BDE-99 (80 µmol/kg bw) exposure resulted in increased liver/bw ratios, but no effects were seen on thyroxine levels [55].

Neurotoxic effects in mice after exposure to PBDEs resulted in permanent aberrations in spontaneous motor behaviour and effected learning and memory functions in rats and mice [46,57-60].

2.3 Human exposure

Concern for adverse effects rose when PBDEs were detected in human blood and tissue samples, as PCBs were a decade earlier [3]. For PCBs, the diet is the main route of exposure, as could be the case for PBDEs but much is still unclear in this respect [19,38,61-63]. Since the PBDEs are also industrial chemicals, direct exposure by inhalation and dermal contact could also be a pathway of human occupational exposure to these chemicals [20].

The average PBDE concentrations detected in various food samples is currently estimated to be around 13 to 113 ng/day, which is equivalent to an estimated maximum human exposure of approximately 2 ng/kg bw/day in Europe and 3 to 4 ng/kg bw/day in the US [27].

Food products that contain the highest amounts of PBDEs are of fatty animal origin e.g. fish and shellfish in European countries and Japan. In Canada and the US the highest concentrations were detected in meat, poultry, and related products.

Levels of PBDEs in human breast milk from Sweden roughly doubled every 5 years in the period from 1972 to 1997, from 0.07 to 4.02 ng/g lipid weight [31], though recently the increase seems to have reached a point where at least BDE-47 is decreasing with peak concentrations found in 1998 [64-66]. In human breast milk samples from 1997 to 2000, levels of BDE-47 ranged from 1.7-2.3 ng/g lipid, with a medium of 2.2 ng/g lipid for people with a diet high in fish, compared to 0.4 ng/g lipid for people with a diet low in fish in Sweden [25]. The exact exposure route for people in North America has not been elucidated yet, but the samples from this continent showed a much higher mean concentration of 34 ng/g lipid in the period 1990 to 2002 [25]. No correlation was observed between age of the individuals and PBDE body burden measured either via blood or milk, nor with body mass index (BMI), alcohol consumption, or computer usage [27,66]. The concentrations of PBDEs in blood were similar to those in milk on a lipid basis. Noticeable, infants in the USA are currently exposed to as much as 120 ng PBDEs/kg bw/day, compared to approximately 10 ng PBDEs/kg bw/day in Germany [27]. In addition, the concentrations of PBDEs in maternal and fetal serum in US samples were reported to be 20 to 106 fold higher than those from Swedish serum samples [67,68]. It should be noticed that in all the above described

studies, the major part of the PBDEs found could be attributed to the lower brominated BDE-47 and in certain individuals BDE-153 With respect to possible adverse effects for humans; no clinical studies have been conducted so far.

3 Fate of PBDEs

The European Union Community banned the use of Penta-BDE and Octa-BDE since August of 2004[1], which was voluntarily followed by the sole US producer (Great Lakes Chemical Corporation, IN, USA) that ceased the production of these PBDE mixtures by the end of 2004. The continued use of Deca- BDE is still under discussion within the United States, but its use is currently not restricted in the European Union[2] and is actually now being declared safe by the EU authorities.
The current ban on Penta- and Octa-BDE mixtures as flame retardants is expected to cause these chemicals to reach a steady state concentration, which will be followed by a decline in biota and abiota. The first indications for this have already been reported in human breast milk samples from Sweden [64-66]. However, due to the persistent nature of PBDEs in the environment, it might take decades before this decline can be observed in all environmental matrices worldwide. In this respect a continuing exposure through older end-product use and waste incineration could still be expected in the coming decades. Furthermore, microbial degradation of BDE-209 in anaerobic sediments and degradation of the compound under the influence of UV-light and sunlight could result into lower brominated congeners [41-43]. Therefore it remains of importance to investigate the possible biological and/or toxicological effects of PBDEs for the years to come, even if these compounds have been banned from the market.

[1] European Union, Restriction of Hazardous Substances Directive (RoHS); Directive 2002/95/EC of the European Parliament and of the Council of 27 January 2003 on the restriction of the use of certain hazardous substances in electrical and electronic equipment, OJ L37, 13 February 2003, page 19

[2] Commission Decision of 13 October 2005 amending for the purposes of adapting to the technical progress the Annex to Directive 2002/95/EC of the European Parliament and of the Council on the restriction of the use of certain hazardous substances in electrical and electronic equipment, OJ L271, 15 October 2005, page 48

BOX 1; Flame retardants

Flame retardants are chemicals that are incorporated into potentially flammable materials such as plastics, rubbers, electronics and textiles to slow down or inhibit the initial phase of a developing fire [3,4]. They are either mixed through a matrix or added to it to prevent burning and ignition. There are various families of flame retardants, as represented in figure 1. The eight manufacturers of PBDEs are located in France, Great Britain, Israel, Japan, The Netherlands and the United States.

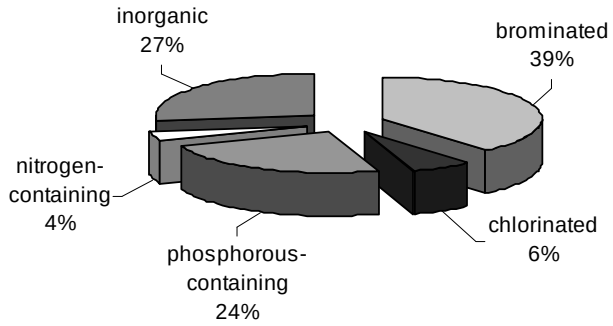

Figure 1; Different families of flame-retardants

Various flame retardancy chemicals have different mechanisms of action. Brominated flame retardants are additive and can reduce the chances of fire by the fact that halogen atoms are released in its thermal decomposition, removing high energy radicals formed during fire [9].
Brominated flame retardants make up 39% of the total market demand, and are currently most effective when cost and performance are considered; other flame retardants require more of the chemicals to obtain the same effect. This might impose a lower burden on the environment due to persistency of some of these compounds, but costs will also increase.

The group of brominated flame retardants can be subdivided into tetrabromobisphenol A (TBBPA), hexabromocyclododecane (HBCD), Penta-BDE, Octa-BDE, and Deca-BDE (figure 2) [1].

BOX 1; Flame retardants -continuation-

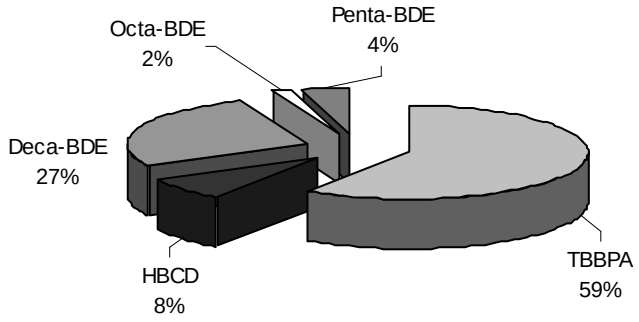

Figure 2; Commercial brominated flame retardants

The commercial Penta-BDE mix contains 24-38% tetra-, 50-60% penta- and 4-8% hexa-brominated BDEs, with penta BDE-99 as predominant congener. The Octa-mix contains 10-12% hexa-, 44% hepta-, 31-35% octa-, 10-11% nona-, and <1% deca-BDE. The major congener is hepta BDE-183. Deca-BDE contains <3% nona- and 97-98% deca BDE-209 [1].

4 Aryl hydrocarbon Receptor

The aryl hydrocarbon receptor (AhR) has been characterized as a ligand-activated transcription factor and is a member of the bHLH/PAS (basic Helix-Loop-Helix/Per-ARNT-Sim) family. The Ah receptor is present in almost every vertebrate, but with large species and tissue differences in sensitivity [69-71]. It binds dioxin-like compounds such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and certain PCBs that can attain a coplanar configuration with high affinity; its endogenous ligand and physiological function are still unknown. Besides planar halogenated polyaromatic compounds such as PCDDs, a large group of structurally very diverse chemicals can also bind to and/or activate AhR-dependent gene expressions [69,70]. The ligand diversity of the AhR suggests that the AhR has a rather promiscuous binding pocket and raises questions as to the spectrum of chemicals that can bind to the AhR [69,70]. Unbound AhR is present in the cytoplasm of the cell as a heterotetrameric 9S complex with two heat shock

Hsp90 protein molecules, as can be seen in figure 3. Following ligand binding, the Hsp90 molecules are released, and the ligand-receptor complex translocates into the nucleus of the cell. There, the AhR binds with a nuclear protein, ARNT (Ah receptor nuclear translocator) [72]. The AhR and ARNT function together as a heterodimer with a high affinity for a specific DNA sequence (5'-GCGTG-3'), the xenobiotic response element (XRE) [73-76]. These XREs have been identified on the 5-prime promotor regions of several AhR inducible genes [77-79]. Binding of the AhR:ARNT complex to the XRE stimulates transcription of adjacent genes, including that of the phase I biotransformation *CYP1A1* gene, encoding the cytochrome P450-1A1 enzyme (CYP1A1) [69,70,77-81].

The structural similarity of certain PBDE congeners to other polyhalogenated aromatic hydrocarbons such as PCBs has raised concerns that these compounds might also act as agonists for the AhR [74]. This aspect of their mechanism of action is still unclear [46,82,83]. However, if certain PBDEs were to act as Ah receptor agonists, this dioxin-like property could warrant inclusion in the toxic equivalence factor (TEF) concept [8,84].

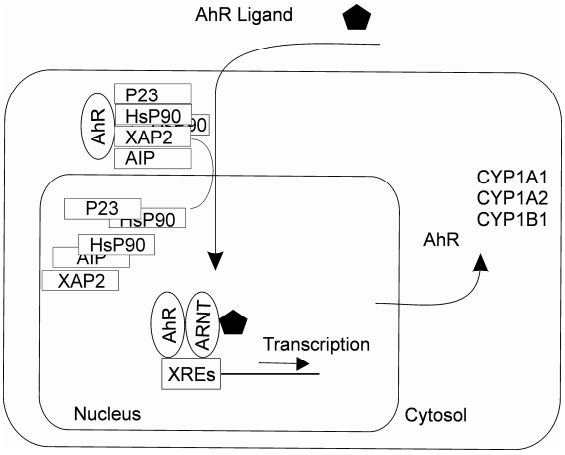

Figure 3; Schematic presentation of the mechanism of AHR-mediated enzyme induction

5 Pregnane X receptor

The human pregnane X receptor (hPXR) is a recently identified orphan nuclear receptor that is activated by a number of compounds that are known *CYP3A4*

inducers [85-88]. The PXR, similarly to its principal target gene *CYP3A4*, is mainly expressed in liver, small intestine, and colon [85,86]. It has an ambiguous ligand binding pocket, which is spherical in shape and hydrophobic, allowing structurally diverse chemicals to bind the receptor [87]. Pharmaceuticals activating PXR include certain antibiotics, barbiturates, glucocorticoids and anti- glucocorticoids, such as rifampicin, phenobarbital (PB), nifedipine, clotrimazole, mifepristone, and metyrapone [89].

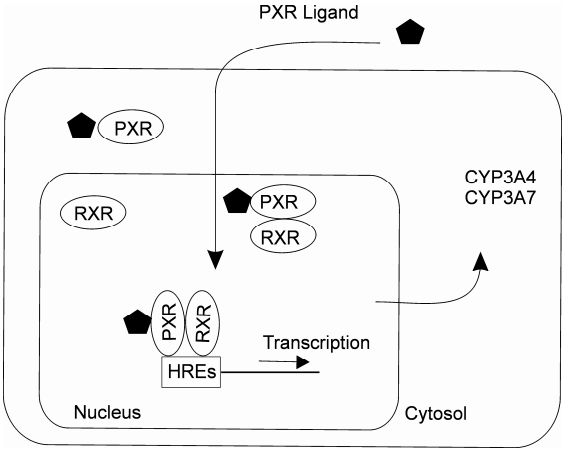

Figure 4; Schematic presentation of the mechanism of PXR-mediated enzyme induction.

Upon ligand binding, hPXR forms a heterodimer with the 9-*cis* retinoic acid receptor-α (RXRα). The PXR:RXRα complex binds hormone responsive elements (HREs) at the 5'-prime promotor region of the *CYP3A4* gene (Figure 4) [85,86,88]. Activation of the *CYP3A4* gene is dependent on the presence of a distal xenobiotic-responsive enhancer molecule (XREM) [88].

There is a substantial overlap between the ligand specificities of PXR and constitutive androstane receptor (CAR), with RXR serving as a common heterodimerization partner [90-92]. PXR and CAR also share ligands, further suggesting the cross-talk between the two receptors [90]. After PXR-null mice were exposed to mouse CYP3A inducers, no induction was observed, which confirms that the PXR is a key element in *CYP3A* induction [92].

PB-like effects include disruption of thyroid hormone homeostasis and thyroid neoplasia [93]. The thyroid hormone is critical for brain development in mammals and thus, disruption of thyroid hormone homeostasis might be related to neurodevelopmental toxicity. Similar effects were observed after rodents were

exposed to non coplanar PCBs and PBDEs [57,94,95]. This might be an indication that some PBDEs are able to elicit PB-like responses at high dose levels.

6 Cytochrome P450

Phase I metabolism involves the hydrolysis, reduction and/or oxidation of xenobiotics [71]. The cytochrome P450 superfamily (CYP P450) enzymes are responsible for most of these phase I reactions, they are involved in the oxidative metabolism of range of structurally diverse xenobiotics, but also endogenous compounds such as steroid hormones, bile acids, fatty acids, and prostaglandins. In addition, P450 enzymes also participate in the detoxification of carcinogens, environmental pollutants, and drugs by conversion to more polar molecules that are excreted more easily from the body [96]. Moreover certain P450 enzymes can bioactivate certain xenobiotics, e.g. polycyclic aromatic hydrocarbons (PAHs) by producing reactive intermediates that are genotoxic. The liver and intestines are important sites for metabolism of endogenous and exogenous compounds.
CYP1A1 is inducibly expressed in all vertebrates although large differences exist in expression levels depending of the type of tissue and species [69-71]. The activity and expression levels of these enzymes depend on the genetic background, age, sex, diet and health status of the individual [71,80]. CYP1A1 enhances the detoxification of lipophilic substrates (many of which are AhR ligands) that might otherwise accumulate to toxic concentrations in a cell. Induction of CYP1A1 only occurs when necessary; upon removal of the stimulus (ligand) by metabolism, CYP1A1 gene expression is no longer upregulated. TCDD is the ligand with the highest binding affinity for the AhR known to date and it is also extremely slowly metabolized by CYP1A1 or any other enzyme. Consequently, the long residence time of TCDD in the cell results in a sustained induction of gene expression and it is thought that most of the potent toxic and biological effects of TCDD and related chemicals are attributed to the prolonged activation of the AhR-dependent gene expression [72]. Use of induction of the CYP1A1 protein and CYP1A1-dependent 7-ethoxyresorufin-O-deethylase (EROD) activity as a sensitive biomarker for AhR-mediated effects has been proposed and applied in the risk assessment for dioxin-like compounds [8].

Another family of cytochrome P450 enzymes, the CYP3A family, is among the most abundant of the mono-oxygenases in the mammalian liver and intestine. Multiple signal transduction pathways might be involved, but the key regulator

seems to be the human pregnane X receptor (hPXR) [97,98]. In contrast to the induction of CYP1A1, the induction of CYP3A enzymes appears to follow less strict structural rules and planar configuration is not a prerequisite. In contrary, non-planar type of compounds e.g. phenobarbital (PB) and certain multiple *ortho* substituted PCBs like PCB-153 seem to have a preference for induction of CYP3A enzymes in humans as well as other mammalian species. In humans, more than 50% of all drugs, as well as other xenobiotics and endogenous substances are metabolized by CYP3A4. Understanding the PB-like induction of CYP3A4 is important for possible clinical drug-drug interactions [99,100].

BOX 2; PCBs

Polychlorinated biphenyls (PCBs) are organohalogen compounds that have been commercially produced since the 1920s for a variety of purposes and belong to the class of persistent organic pollutants (POPs). They were extensively used in industrial applications e.g. because of their insulating and flame retardancy properties, which resulted in a wide-spread distribution of PCBs depending on their structural and physio-chemical properties and the degree of chlorination [2]. Low water solubility, high lipid solubility and slow biological degradation are properties that lead to persistence and accumulation in lipid rich body tissues and fluids from both humans and wildlife species. They were banned in open applications in many countries since the 1970s due to their persistent nature and potent toxic effects [4,5]. PCB exposure, in occupational as well as environmental settings, has been associated with e.g. chloracne, carcinogenesis, teratogenesis, impaired immune responses, endocrine disruption, as well as the induction of certain enzymes like cytochrome P450-1A1 (CYP1A1), -2B (CYP2B), and -3A (CYP3A). CYP1A1 induction is a sensitive biomarker that also correlates with adverse health effects of dioxin-like compounds from which 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is considered the most toxic congener [4,6,10]. Concerns for PCBs arose after an environmental accident in 1973 with brominated biphenyls (PBBs) that are strongly related to PCBs. Cattle feed in Michigan was contaminated with the flame retardant PBB and resulted in a widespread distribution in humans through contaminated meat and dairy products [15].

BOX 2; PCBs -continuation-

There are 209 possible PCBs congeners and their biological and toxic effects depend on their chlorination pattern (Figure 5) [4,6,7,10,28-30].

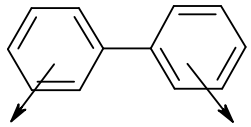

Figure 5; Structure of polychlorinated biphenyls (PCBs) with (x+y≤ 10)

PCBs have been structurally divided into three groups; non-*ortho* PCBs with dioxin-like properties and a planar configuration, mono-*ortho* substituted PCBs with some dioxin-like properties, and multiple-*ortho* substituted PCBs with a non-planar configuration and no or very low affinity for the AhR, consequently lacking dioxin-like effects [6-8].

Current human exposure to PCBs and dioxin-like compounds occurs primarily through dietary intake of lipid rich food (mainly from animal origin) or occupational exposure [11-14]. Though concentrations of PCBs and dioxins have clearly declined over the last decades, the release into the environment is expected to continue for quite some time due to leakage of PCBs from old equipment, building materials, and landfills sites [6,23-26].

7 Scope and objectives of this thesis

The structural similarity of certain polybrominated diphenyl ethers (PBDE) with other polyhalogenated aromatic hydrocarbons such as polychlorinated biphenyls (PCBs) has raised concerns that these PBDE congeners could act as agonists for the aryl hydrocarbon (Ah) receptor.

Studies in this thesis describe the possible interaction between the most environmentally relevant PBDEs (BDE-47, -99, -100, -153, -154, -183, and BDE-209) and the AhR-mediated CYP1A1 induction by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in various mammalian *in vitro* models (Figure 6). BDE-77 is not environmentally relevant but resembles PCB-77 due to its lack of *ortho*-bromine and two adjacent bromine atoms on both aromatic rings. Therefore it was also included in these experiments as it was expected to have the most significant AhR agonistic properties, if present at all. All PBDEs used in this study have been extensively purified by a clean-up method using activated carbon in order to remove any impurities like brominated dibenzofurans that might have interaction with the AhR and could cause false-positive results.

We have assessed the possible AhR-agonistic and AhR-antagonistic effects of these highly purified PBDEs in human breast carcinoma (MCF-7), human hepatoma (HepG2), and rat hepatoma (H4IIE) cells (Chapter two). These cell lines have been commonly used in the past for mechanistic studies with AhR ligands [101]. CYP1A1 is the major enzyme that catalyses the deethylation of 7-ethoxyresorufin to resorufin [102] and this 7-ethoxyresorufin-O-deethylation (EROD) activity was therefore used as a marker for CYP1A1 activity.

Clearly, primary cell cultures of hepatocytes provide a more physiologically relevant and possibly more sensitive system than cell lines since they originate directly from the body [103-105]. Therefore, the possible AhR agonistic and AhR antagonistic effects of these PBDEs were also studied in primary hepatocytes of cynomolgus monkey *(Macaca fascicularis)* with again EROD activity as a marker for CYP1A1 activity (Chapter three).

The observed interactive effects between dioxin-like compounds and PBDEs described in chapter two and three lead to the assumption that PBDEs could actually interfere with the AhR-mediated pathways. However, whether this occurred at receptor-binding level or at the level of the DNA remained unanswered. In chapter four the Ah receptor pathway was studied more closely, in addition to measuring CYP1A1 activity. Two stably transfected rodent hepatoma cell lines were used, containing an AhR-responsive enhanced green fluorescent

protein (EGFP) reporter gene with approximately 500 bp of the CYP1A1 promoter, including 4 XRE sequences (mouse Hepa1c1c7, rat H4G1.1c2) [106,107]. In addition, a human hepatoma (HepG2) cell line containing either a stably transfected construct of two isolated XRE sequences in front of a heterologous promoter (XRE-HepG2) or transiently transfected with a Gal4-AhR construct, were also used to investigate the AhR and XRE binding by PBDEs further.

Besides dioxin-like properties of PBDEs, the PB-like properties were examined by looking at the possible induction of CYP3A4 through the human pregnane X receptor (hPXR) in transiently transfected HepG2 cells (Chapter five).

Furthermore, we stress the need for clean-up procedures when testing singular chemicals for their biological or toxicological responses. Obviously species and tissue differences could vary the outcomes of the experiments mentioned above, but small impurities with AhR inducers could alter the outcome of these *in vitro* experiments completely. Chapter six describes a study to assess the ability of highly purified environmentally relevant mono-*ortho* (MO-) PCBs (PCB-105, -118, -156, and -167) to bind the AhR, as well as induction of CYP1A1 using two stably transfected rodent cell lines as described above. In contrast to PBDEs, these MO-PCBs have been assigned TEF values for dioxin-like compounds and have been tested repeatedly in the past. Using the same rigorous clean-up procedure and AhR dependent *in vitro* assays as with PBDEs, additional experiments with this environmental relevant group of compound were performed to further validate existing TEF values.

A summary and general discussion of the results is given in chapter seven.

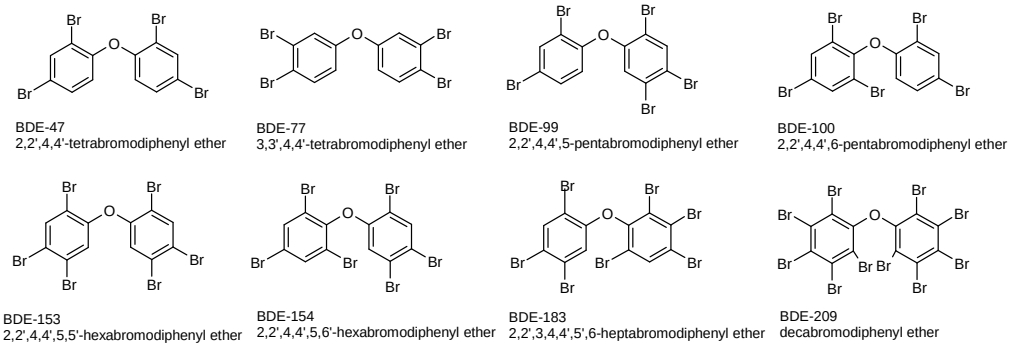

Figure 6; Structures of the PBDE congeners used in our studies, BDE-47, -77, -99, -100, -153, -154, -183, and -209

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CHAPTER TWO

Effects of Polybrominated Diphenyl Ethers (PBDEs) on Basal and TCDD-Induced Ethoxyresorufin (EROD) Activity and Cytochrome P450-1A1 Expression in MCF-7, HepG2 and H4IIE Cells

A.K. Peters, K. van Londen, Å. Bergman, J. Bohonowych, M.S. Denison,
M. Van den Berg, J.T. Sanderson
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Abstract

Polybrominated diphenyl ethers (PBDEs) are used as additive flame-retardants in consumer products to reduce the chances of ignition and burning. Levels of certain PBDE congeners have been increasing in fish, wildlife, and human tissues during the last decades. Some PBDEs are lipophilic and persistent, resulting in bioaccumulation in the environment. The structural similarity of PBDEs to other polyhalogenated aromatic hydrocarbons such as PCBs has raised concerns that PBDEs might act as agonists for the aryl hydrocarbon receptor (AhR). To study the possible AhR-mediated effects of the environmentally relevant PBDEs (BDE-47, -77, -99, -100, -153, -154, -183, -209), the induction of cytochrome P450-1A1 (CYP1A1) was studied in human breast carcinoma (MCF-7), human hepatocellular carcinoma (HepG2), and rat hepatoma (H4IIE) cells. 7-Ethoxyresorufin-O-deethylase (EROD) was used as a marker for CYP1A1 activity. Cells were exposed for 72 hours to various PBDE concentrations (0.01-10 μM). Positive controls were 2,3,7,8-TCDD (0.001-2.5 nM) and PCB-126 (0.01-10 nM). None of these PBDEs were capable of inducing EROD activity; this was confirmed by quantitative RT-PCR for CYP1A1 mRNA. However, in cells exposed to PBDEs in combination with TCDD, a concentration-dependent decrease in TCDD-induced EROD activity occurred. Co-exposure of BDE-153 (10 μM) and a maximally inducing concentration of TCDD (1 nM) reduced EROD activity to 49% of the maximum induction by TCDD alone. All tested PBDEs showed similar effects in each cell line, though quantitative differences were observed. The observed decrease in CYP1A1 activity was not due to PBDE-dependent catalytic inhibition of EROD activity or cytotoxicity, nor were decreased CYP1A1 mRNA levels observed. However, inhibition of luciferase induction in mouse (Hepa) and rat (H4IIE) hepatoma cells containing a stably transfected AhR-responsive luciferase reporter gene, suggests that BDE-77 is a weak AhR antagonist or partial agonist.

Introduction

Flame-retardants are added to materials to reduce the emission of heat and carbon monoxide in fires. Polybrominated diphenyl ethers (PBDEs) are used as additive flame-retardants in plastic materials, paints, and textile fabrics. Some PBDEs are lipophilic and persistent, and consequently bioaccumulate [1,2]. There are three commercial mixtures of PBDEs in use (Penta-BDE, Octa-BDE, and Deca-BDE), with a global estimated demand close to 77.000 metric tonnes in 1999 (Bromine Science and Environmental Forum, 2000). During the last decades, levels of PBDEs increased in fish, wildlife, and in human blood [3-7], adipose tissue [8] and milk samples. Levels of PBDEs in human milk doubled every 5 years in the period from 1972 to 1997, from 0.07 to 4.02 ng/g lipid weight [9], though recently the increase seems to have reached a point where at least BDE-47 is decreasing [6,7]. The structural similarity of certain PBDE congeners to other polyhalogenated aromatic hydrocarbons such as polychlorinated biphenyls (PCBs), has raised concerns that these compounds might act as agonists for the aryl hydrocarbon (Ah) receptor [10]. This aspect of their toxicology is still unclear [11-13]. However, if certain PBDEs were to act as Ah receptor agonists, they would warrant inclusion in the toxic equivalence factor (TEF) concept [14,15].

The Ah receptor is a ligand-dependent nuclear receptor that is present in almost every vertebrate. The receptor binds dioxin-like compounds such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and certain PCBs that can attain a coplanar configuration with high affinity; its endogenous ligand and physiological function are still unknown. The unbound Ah receptor is present in the cytosolic compartment of the cell as a multiprotein complex. Following ligand binding, the ligand-receptor complex translocates into the nucleus of the cell, the AhR dissociates from the complex and binds to a nuclear protein ARNT (Ah receptor nuclear translocator). This newly formed complex has a high affinity for a specific DNA sequence (Dioxin Responsive Elements), and formation of the AhR:ARNT:DRE complex results in an increase in the transcription of various genes, including that of the *CYP1A1* gene, encoding the cytochrome P450-1A1 enzyme (CYP1A1) [16]. CYP1A1 is involved in phase I biotransformation of xenobiotics and endogenous compounds such as estrogens. The CYP enzymes can detoxify xenobiotics or bioactivate them by producing reactive intermediates. Although CYP1A1 is expressed in all mammals, there are large differences in expression levels among species [17-19].

The objectives of the present study were to assess the possible AhR-mediated effect of the most environmentally relevant PBDEs: BDE-47, -99, -100, -153, -154, -183, and BDE-209 [5,20] (see Table 1). BDE-77 was included due to its lack of *ortho*-bromine and hence structural resemblance to PCB-77. To determine the possible AhR-agonistic and AhR-antagonistic effect of these PBDEs, the Ah receptor-mediated induction of the CYP enzyme 1A1 was studied in human breast carcinoma (MCF-7), human hepatoma (HepG2), and rat hepatoma (H4IIE) cells. These cell lines have been commonly used in the past for mechanistic studies with AhR ligands [21]. CYP1A1 is the major enzyme that catalyses the deethylation of 7-ethoxyresorufin to resorufin [22]. This ethoxyresorufin-O-deethylation (EROD) activity was used as a marker for CYP1A1 activity.

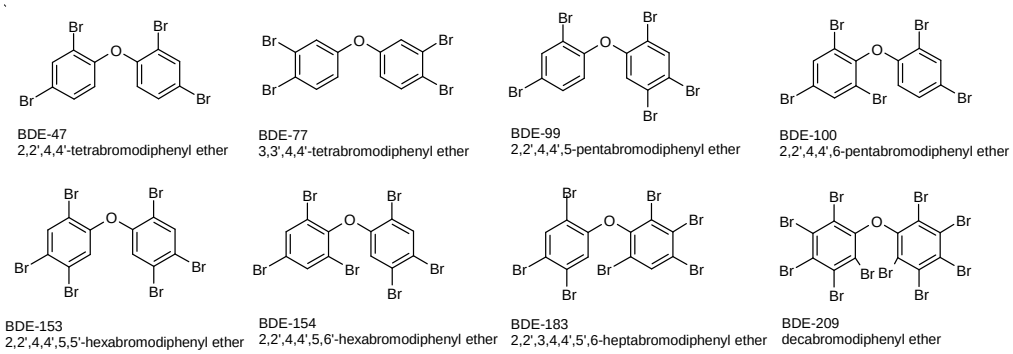


Table 1; Molecular structures of PBDEs. The most environmentally relevant PBDEs (BDE-47, -99, -100, -153, -154, -183, and BDE-209) and BDE-77

Materials and methods

Chemicals

The chemicals used were obtained from the following companies: 2,3,7,8-TCDD (>99% pure), Cambridge Isotope Laboratories (Woburn, MA, USA); 3,3',4,4',5-pentachlorobiphenyl (PCB-126) (>98% pure) was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). PBDE congeners (>98% pure) were synthesized and each congener was subjected to a specific purification on activated charcoal and Celite to remove possible contamination with dioxin-like compounds such as PBDFs [23]. The cell culture media (DMEM) was obtained from Gibco BRL (Breda, The Netherlands), alpha minimal essential media (αMEM) from Gibco (Carlsbad, CA, USA), and fetal calf serum from Atlanta Biologicals (Atlanta, Georgia, US).

Cell culture lysis reagent and stabilized luciferase substrate were obtained from Promega (Madison, WI, USA). Chemicals used for the TaqMan quantitative one-step RT-PCR were obtained from PE Applied Biosystems (Nieuwerkerk a/d IJssel, The Netherlands), RNA insta-pure from Eurogentec (Seraing, Belgium), and all other chemicals were obtained from Sigma Chemical Company (St. Louis, MO, USA).

Cell lines and cell culture

The MCF-7 and H4IIE cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and HepG2 cells from Deutsche Sammlung von Microorganismen und Zellkulturen (DSZM, Braunschweig, Germany). MCF-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 0.01 mg/ml insulin, 100 µg/ml penicillin, 100 µg/ml streptomycin and 5% fetal calf serum. The H4IIE and HepG2 cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 100 µg/ml penicillin, 100 µg/ml streptomycin, and 10% fetal calf serum. The cells were cultured in a humidified atmosphere with 5% CO_2 at 37°C.

Cell viability assays

MTT assay

Mitochondrial capacity to reduce MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan [24] was used as a measure of cell viability. Cell cultures were incubated with TCDD (0.001-2.5 nM), PCB-126 (0.01-10 µM) or BDE-47, -77, -99, -100, -153, -154, -183, -209 (0.01-10 µM) for 72 hours, followed by incubation with serum-free medium containing 1 mg MTT/ml for 30 minutes at 37°C. After this MTT solution was removed, the cells were washed with warm PBS (37°C). The formazan was extracted from the cells with 1 ml isopropanol and incubated for 10 min at room temperature, formazan concentration was determined spectrophotometrically at 560 nm. Cell viability was calculated using DMSO treated cells as the 100% viable control.

Lactate dehydrogenase (LDH) assay

Plasma membrane integrity was determined by measuring LDH leakage into the culture medium [25]. The reduction of NADH in the presence of pyruvate was measured in the culture medium of cells that had been exposed to the test chemicals for 72 hours. In one cuvet 100 µl medium, 1 ml phosphate buffer containing 66 mg/l pyruvate and 20 µl NADH were added and measured spectrophotometrically at 340 nm (every 0,4 sec during 20 sec at room

temperature). Controls were performed with 0.1% (w/v) Triton X-100 and set as 100% LDH release.

Alamar Blue assay

Metabolic activity results in the chemical reduction of the blue-colored Alamar Blue (AB) dye to its fluorescent red form [26]. Cells that had been incubated with the test chemicals for 72 hours as described before, were washed with warm PBS (37°C), and serum-free medium (500 µl) containing 10% AB was added. After 2 hours, the fluorescence of the media was measured at 37°C (excitation wavelength 530 nm, emission wavelength 590 nm). Metabolic activity was calculated using DMSO treated cells as the 100% viable control.

EROD assay

Ethoxyresorufin-O-deethylation (EROD) activity was used as a marker for CYP1A1 activity, using a modification of the method described by Burke and Mayer [22,27]. MCF-7 cells were seeded in 24-well plates at a density of $1x10^5$ cells/well; HepG2 and H4IIE cells were seeded at a density of $2x10^4$ cells/well. To assess the agonistic activity of PBDEs, cultures of MCF-7, HepG2, and H4IIE cells were exposed to increasing concentrations of the PBDEs (0.01-10 µM), the positive controls TCDD (0.001-2.5 nM), and PCB-126 (0.01-10 nM), or the vehicle control DMSO (0.1%). To determine antagonistic effects, cells were exposed to mixtures of TCDD (0.001-2.5 nM) and PBDEs (0.01-10 µM). After 72 hours, medium was removed and the cells were washed twice with warm PBS (37°C) and serum-free medium containing 5 mM $MgCl_2$, 5 µM 7-ethoxyresorufin, and 10 µM dicumarol was added to each well. The conversion of ethoxyresorufin to resorufin, which has an excitation and emission wavelength of 530 nm and 590 nm respectively, was followed fluorometrically at 37°C over a 10 min period.

Quantitative RT-PCR

MCF-7 and HepG2 cells were exposed for 72 hours to positive controls (TCDD 0.001-2.5 nM, PCB-126 0.01-10 nM), vehicle controls (DMSO 0.1% or 0.2%) and the indicated PBDEs (0.01-10 µM) with and without co-exposure to TCDD (1 nM) in 12-well plates.

RNA was isolated using the RNA Insta-Pure System according to the manufacturer's instructions. The amount of RNA was quantified spectrophotometrically (260/280 nm) and checked for DNA impurities with 3% agarose gel electrophoresis and ethidium bromide staining. Quantitative comparison of CYP1A1 mRNA levels was carried out using real-time PCR

technology with beta-actin as the endogenous control. Primers and probes for CYP1A1 (forward primer 5'-AGC GGA AGT GTA TCG GTG AGA; reverse primer 5'-CTG AAT TCC ACC CGT TGC A) and beta-actin (forward primer 5'-TCC TCC TGA GCG CAA GTA CTC; reverse primer 5'-CTG CTT GCT GAT CCA CAT CTG) were designed using Primer Express software (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Probes for CYP1A1 (5'-TGC CCG CTG GGA GGT CTT TCT CTT) and beta-actin (5'-TGG CCT CGC TGT CCA CCT TCC A) were designed and labeled at the 5'-end with a reporter dye (VIC and FAM, respectively) and a quencher dye (TAMRA) at the 3'-end. Total RNA (10 ng) from the cells was amplified using a Taqman thermal cycler (7000 Sequence Detection System, ABI PRISM® Applied Biosystems). One-step RT-PCR Mastermix was used under the following conditions: 30 min at 48°C, 10 min at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 min at 60°C. Fluorescence data were processed and analyzed with ABI PRISM® Sequence Detection software (Applied Biosystems). The results of the PCR assay were expressed as $C_{T\ Beta\ actin}$ (number of cycles needed to generate a fluorescence signal above a pre-defined threshold for beta actin) divided by $C_{T\ CYP1A1}$. Induction of CYP1A1 mRNA was determined by comparison to the DMSO control. The RT-PCR amplification products were checked for correct band size using a 1% agarose gel electrophoresis and ethidium bromide staining.

Recombinant Cell Lines, Chemical Treatment and Measurement of Luciferase activity

Mouse hepatoma (H1L1.1c2) and rat hepatoma (H4L1.1c4) cells containing a stably transfected TCDD- and AhR-responsive firefly luciferase reporter gene plasmid were prepared and grown as previously described [28]. These cells respond to TCDD and other AhR agonists with the induction of luciferase gene expression in a time-, dose-, chemical- and AhR-specific manner [27-29]. Cells were seeded in sterile, white, clear-bottomed 96-well plates at $7.5x10^4$ cells/well in 100 µl αMEM containing 10% fetal calf serum and incubated at 37°C for 12 hours prior to treatment. For measurement of agonist activity, cells were treated with DMSO, TCDD (1 nM) or the indicated concentration of PBDE. For measurement of antagonist activity, cells were treated with DMSO, TCDD (1 nM), or TCDD (1 nM) and PBDE at the indicated concentration. Following incubation with the indicated chemical(s) for 4 hours at 37°C, all wells were washed twice with PBS (100 µl), then 50 µl of Promega Lysis buffer added to each well and the plate was shaken for 15 minutes to lyse the cells. Luciferase activity was measured using an automated

microplate luminometer (Dynatech ML3000; Chantilly, VA) in enhanced flash mode with the automatic injection of 50 µl of Promega stabilized luciferase reagent.

Other Assays

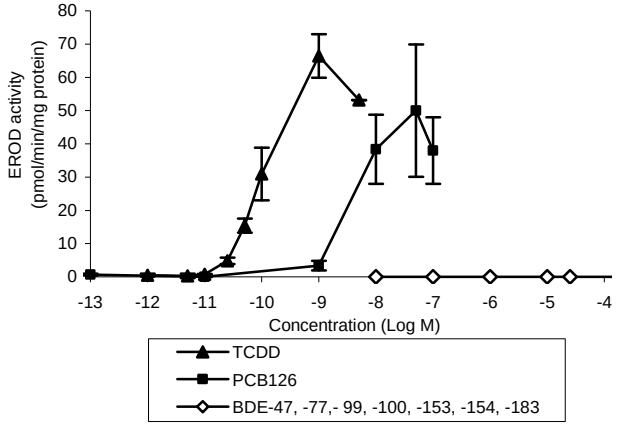
Protein concentrations in separate wells were determined using the method of Lowry [30], using bovine serum albumin as a protein standard. The catalytic activities of CYP1A1 were corrected for the amount of protein per well, to allow for a better comparison among assays.

Results

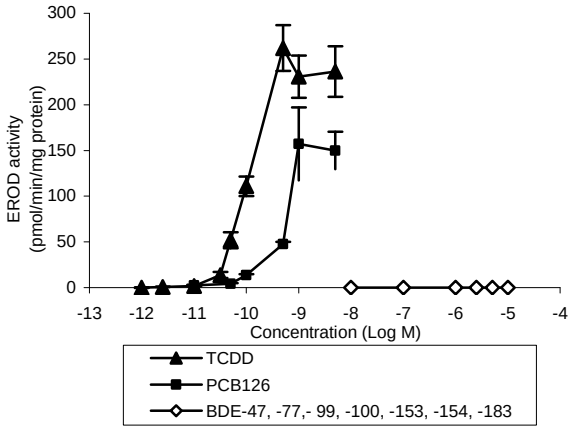
Effects of PBDEs on EROD activity and CYP1A1 mRNA levels

There was a concentration-dependent increase in EROD activity measured after a 72h exposure to the positive controls TCDD and PCB-126. EC_{50} values for TCDD were 0.40, 0.08, and 0.01 nM in MCF-7, HepG2 and H4IIE cells respectively. EC_{50} values for PCB-126 were 2.5, 0.5, 0.1 nM respectively. Cells treated with PBDEs (BDE-47, -77, -99, -100, -153, -154, -183) did not show any induction of EROD activity and thus EC_{50} values could not be calculated (see Figure 1 A, B, C). This lack of induction was also confirmed in both human cell lines MCF-7 and HepG2 by quantitative RT-PCR. The $C_{T\ beta\ actin}/C_{T\ CYP1A1}$ values of PCB-126 (1 nM) were 77% of TCDD (1 nM) in both the MCF-7 as the HepG2 cell line. After validation curves for stepwise dilutions of the mRNA of both human cell lines, both the CYP1A1 and beta-actin curves had a slope for which applied $E=10^{-1/slope}$, with E being approximately 2. The expression of the endogenous control beta-actin was not affected by any of the treatments.

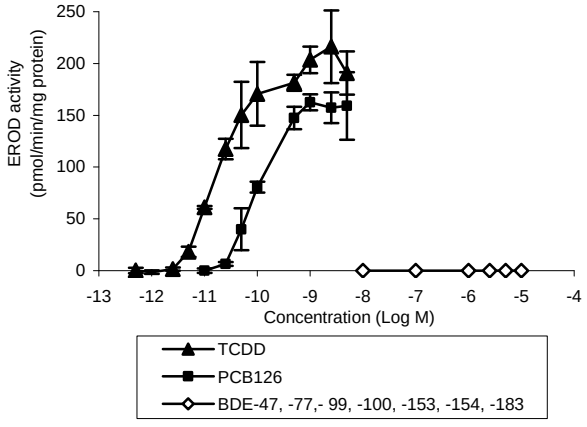
In contrast to their inability to induce EROD activity, co-incubation of PBDEs with TCDD for 72h, resulted in a concentration-dependent decrease in TCDD-induced EROD activity. The positive control TCDD produced maximal EROD induction at 1 nM, which was set at 100% efficacy. In the presence of BDE-153 at concentrations of 1 and 10 µM, the induction efficacy of TCDD in MCF-7 cells was reduced to 75% and 49% of the maximum (Figure 2A).



1A

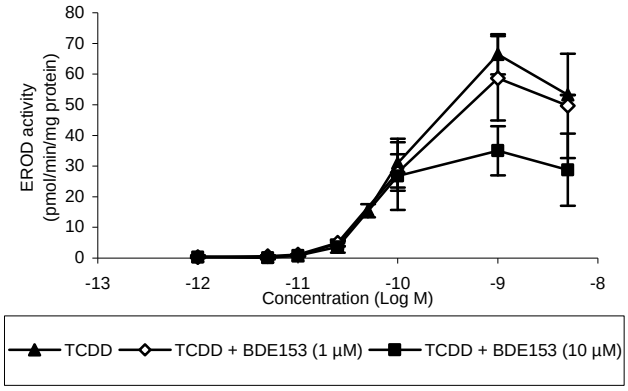


1B

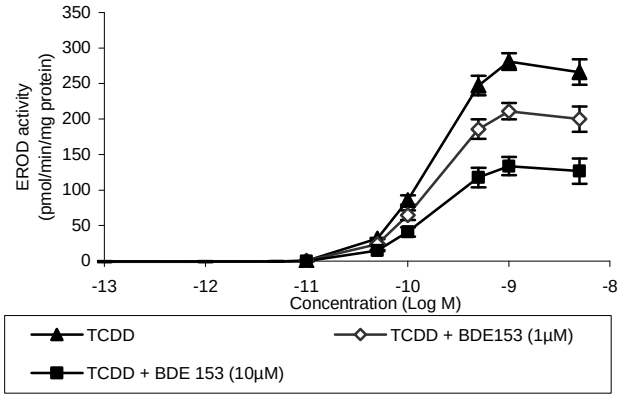


1C

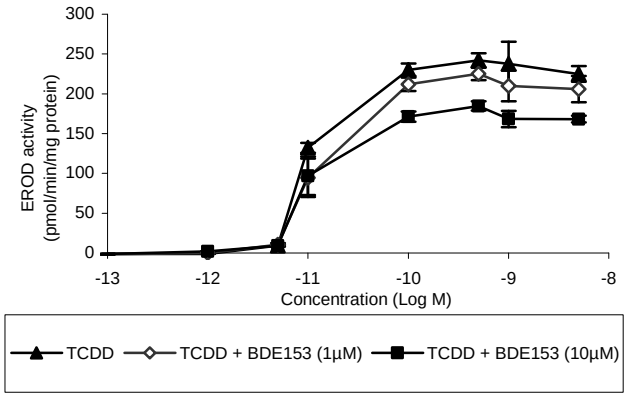
Figure 1 A, B, C; Induction of EROD activity in MCF-7 (A), HepG2 (B), and H4IIE (C) cells, with exposure to both positive controls TCDD (0.001-2.5 nM) and PCB-126 (0.01-10 nM) and BDE-47, -77, -99, -100, -153, -154, -183 (0.01-10 µM). The data are expressed as mean ± standard deviation (n=3).



2A



2B



2C

Figure 2 A, B, C; Examples of effects of co-exposure with PBDEs (1, 10 µM) on TCDD-mediated induction of EROD activity in MCF-7 (A), HepG2 (B), and H4IIE (C) cells. All PBDEs tested showed similar inhibitory effects on EROD-activity after co-exposure, though quantitative differences were observed. The data are expressed as mean ± standard deviation (n=3).

Inhibition of TCDD-dependent EROD induction was also observed in HepG2 and H4IIE cells (Figure 2B, C). All PBDEs tested (BDE-47, -77, -99, -100, -153, -154, -183) caused a similar decrease in TCDD-induced EROD activity, although not always statistically significant (see Table 2). BDE-209 could not be tested in the concentration range where other PBDEs showed inhibitory effects, due to its insolubility (>0.5 μM).

When the RNA isolated from MCF-7 and HepG2 cells exposed to various concentrations of PBDEs in combination with TCDD (1 nM) was amplified in a quantitative RT-PCR assay, there was no statistically significant difference between the C_T values from co-exposed cells and TCDD-exposed cells. Thus, no differences in mRNA levels of CYP1A1 were found.

The observed decrease in TCDD-induced EROD activity was not caused by cytotoxicity since there was no decrease in MTT reduction after co-exposure in MCF-7, HepG2 and H4IIE cells as compared to controls. This lack of cytotoxicity was confirmed by LDH and Alamar Blue assays (data not shown).

To assess whether the PBDEs inhibitory effect was due to direct catalytic inhibition of EROD activity, we examined the ability of the PBDEs to inhibit the TCDD-induced EROD activity in cells that had been exposed for 72 hours to the maximal inducing concentration of TCDD (1 nM). In these experiments, PBDEs (0.01-10 μM) were added to the cells 5 minutes prior to measurement of EROD activity. Under these conditions, the PBDEs were not able to reduce TCDD-induced EROD activity, while the positive control alpha-naphthoflavone (ANF, 1 μM), a known inhibitor of CYP1A1 activity, did reduce the TCDD-induced EROD activity.

Since it is possible that the relative affinity of 7-ethoxyresorufin is significantly greater than that of the PBDEs, the assay was repeated with various concentrations of the substrate 7-ethoxyresorufin (0-5 μM) during the assay as previously described [31], with alpha-naphthoflavone (ANF, 0-1 μM) as positive control. Similar to the results above, no catalytical inhibition was found for any PBDE although ANF was able to decrease the TCDD-induced CYP1A1 activity significantly with decrease at all concentrations tested (data not shown). Together, these results suggest that PBDEs do not reduce CYP1A1 activity via a competitive CYP1A1 substrate binding mechanism.

Another possibility for the observed inhibition is antagonism at the level of the AhR and/or AhR signaling pathways. To examine this possibility, experiments were carried out using mouse hepatoma (H1L1.1c2) and rat hepatoma (H4L1.1c4) cells. These cells contain a stably transfected AhR-responsive luciferase reporter gene that responds to AhR binding with luciferase induction in a dose-,

temperature and chemical specific manner [27-29]. Of the PBDEs that were tested (BDE-47, -77, -99, -100, -154, BDE-153, and BDE-183), only BDE-77 showed a concentration-dependent and statistically significant ($p<0.05$) inhibitory effect after co-exposure with TCDD (1 nM) (see Figure 3).

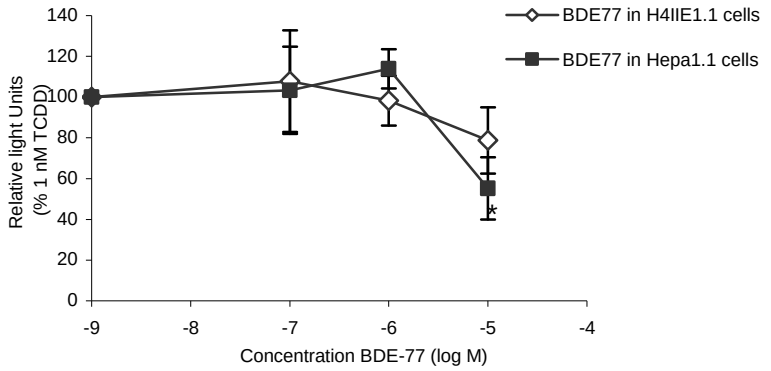


Figure 3; Effects of co-exposure of BDE-77 (0.1-10 µM) with TCDD (1 nM) in recombinant AhR-responsive luciferase-transfected mouse hepatoma (H1L1.1c2) and rat hepatoma (H4L1.1c4) cells. The data is expressed as mean ± standard deviation (n=3). *: significantly lower than the response to TCDD alone ($p<0.05$).

Discussion

Are PBDEs agonists of the Ah receptor?

In previous studies, it has been reported that several PBDEs competitively bind the Ah receptor and induce responses such as CYP1A1 mRNA, CYP1A1 protein and EROD induction as well as an AhR responsive luciferase reporter gene, suggesting a dioxin-like behavior [32-34]. Chen and Bunce (2003) reported weak AhR binding of PBDEs; the environmentally relevant BDE-47 and BDE-99 were among the least active, while BDE-77 activated the AhR-DRE complex formation of rat cytosolic AhR at 60% of that of TCDD and induced CYP1A1 protein in their studies.

In our studies with the environmentally relevant PBDEs (BDE-47, -99, -100, -153, -154, -183, -209) and BDE-77 no such induction of CYP1A1 mRNA or EROD was observed in the AhR responsive MCF-7, HepG2 and H4IIE cell lines. Chen et al [32] also found no induction of CYP1A1 after the EROD assay in several cell culture systems for BDE-47, -99, -154. However, they also reported weak induction of CYP1A1 activity by BDE-77, -100, -153, -183, consistent with weak to medium DRE

binding. After a DR-CALUX assay, Behnisch and co-workers [33] found similar results; environmentally relevant PBDEs showed very weak EROD induction.
In commercial mixtures of PBDEs (Penta-BDE, Octa-BDE, Deca-BDE), low levels of contamination by polybrominated dibenzofurans and polybrominated dibenzo-p-dioxins (PBDF, PBDD) may occur [35]. These compounds bind the AhR with high affinity and induce AhR-dependent gene expression. If these impurities remain in the PBDE samples after purification, they may be responsible for part of the observed CYP1A1 induction in exposed cell systems that is otherwise attributed to the PBDEs. Thus, it is highly important to rule out the absence of these polybrominated dibenzofurans and dioxins when studying biological or toxicological properties of individual PBDEs or their commercial mixtures. Such contamination was likely present in our initial BDE-77 preparation that showed some CYP1A1 induction. However, when highly purified BDE-77 from the Wallenberg laboratory was used, this induction was no longer detected. This effect has also been seen by Koistinen et al when conducting assays with polychlorinated diphenylethers (PCDEs) [36]; they showed that even a small contamination with PCDFs (less than 1% by weight) almost entirely explained the observed induction by these PCDEs.
We conclude that the PBDEs used in our study are not able to activate the AhR, and consequently are not able to induce CYP1A1 enzyme activity and/or CYP1A1 mRNA in the human MCF-7 and HepG2 and rodent H4IIE cancer cell lines. If a compound does not bind to and activate the AhR, the compound need not be assigned a TEF value [14,15]. The absence of CYP1A1 induction by the environmentally relevant PBDEs tested in our study supports the increasing body of evidence that these compounds do not require inclusion in the TEF concept for dioxin-like compounds.

Are PBDEs antagonists of the Ah receptor?
After exposure of the cells to mixtures of TCDD and PBDEs, a significant decrease in TCDD-induced EROD activity was observed. Cytotoxicity and catalytic inhibition could be ruled out as possible causes of the observed decrease. However, it should be noted that there was no decrease in CYP1A1 mRNA levels after co-exposure of the cells to mixtures of TCDD and PBDEs. A similar possible antagonistic effect at the level of catalytic activity was observed previously for PCB-153 [27] and for BDE-47, BDE-99 (strong) and BDE-77, -100, -126, -153, -156 (weak) [32,34]. Chen and Bunce (2003) also found that PBDEs did not change mRNA levels after co-exposure with TCDD even though an inhibitory effect was seen on

EROD activity. One of the suggested mechanisms is that a low level of activated nuclear AhR by TCDD is enough to maintain the transcription of the CYP1A1 gene and would therefore not result in a difference in mRNA CYP1A1 level. This however does not explain the decreased EROD activity in our study and that of Chen and co-workers [34]. Another explanation might be that PBDEs interfere with other post transcriptional processes such as heme synthesis as previously suggested for PCB-77 [37]. It has also been suggested that PHAHs are able to cause a dose-dependent increase in porphyrines [38], which are precursors of heme.

We addressed this problem of mixture interaction by a luciferase assay in recombinant mouse hepatoma (H1L1.1c2) and rat hepatoma (H4L1.1c4) cells, only BDE-77 (10 µM) was able to exert a significant concentration-dependent antagonistic effect on AhR-mediated luciferase induction after co-exposure with TCDD (1 nM) ($p<0.05$). BDE-77 was included in our study because of structural resemblance to PCB-77 and potential to attain a relatively coplanar configuration. It is well known that some planar PCBs bind to the AhR with strong affinity [39]; binding of BDE-77 in these experiments to the AhR might indicate a structure activity similar for AhR binding with PCBs for this specific PBDE congener, though with much weaker affinity. Clearly, the mechanism by which the PBDEs are able to inhibit TCDD-induced CYP1A1 activity needs to be investigated further.

The question remains whether this inhibitory effect by PBDEs on dioxin-like activity also applies to more toxicological relevant parameters besides CYP1A1 activity. Their inability to exert AhR-dependent effects does not mean that these chemicals cannot produce other adverse effects at these levels through different modes of action, such as neurotoxicity [40,41]

In addition, it should also be noted that the observed inhibitory effects by PBDEs were predominantly observed at relatively high levels of TCDD, which may have little relevance for the actual European background exposure situation of the human population, but might be more important for occupational and North-American situation. The concentration range in which the individual PBDEs were tested effectively in our research (0.5 µg/g up to 7.2 µg/g; 1 µM tetrabromodiphenyl ether and 10 µM heptabromodiphenyl ether respectively) exceed the total PBDE concentration found in European human blood (3.3 ng/g lipid weight [42]), human breast milk (4 ng/g lipid weight [9]) and in human adipose tissue (11.7 ng/g lipid weight [43]). The ratio PBDE/TCDD that we tested in our research ($1.5x10^3$ up to $22x10^3$) is in the same order of magnitude and up to a thousand-fold higher as the ratio of PBDE and TCDD in human blood ($1.4x10^3$ [42,44]). The data from literature is composed of total PBDE concentration,

while we use singular congeners. Furthermore, we must take into account that effects on the general population will be lower due to the biological availability of the compounds and binding to proteins.

Based on the results obtained with PBDEs in this study we see no arguments to support inclusion of these compounds in the TEF concept for dioxin-like compounds. Further research is necessary to assess the full scope of possible toxic effects of PBDEs since some of them are still in use commercially and their levels in the environment remain relatively high.

Inhibitory effects of PBDEs on TCDD-induced EROD activity

Compound	Concentration	MCF-7	sd	HepG2	sd	H4IIE	sd
TCDD	1 nM	100	± 10.2	100	± 12.8	100	± 7
BDE-47	1µM	74.3*	± 1.13	91.7	± 7	91.5	± 2.26
	10µM	40.6*	± 4.67	72.5	± 13.65	50.1*	± 3.11
BDE-77	1µM	68.5*	± 1.3	57.6*	± 8.26	50.0*	± 19.83
	10µM	36.5*	± 24.83	37.3*	± 5.95	20.4*	± 1.37
BDE-99	1µM	90	± 3.13	81.2	± 7.72	98.9	± 1.63
	10µM	52.3*	± 2.87	84.2	± 15.71	58.4*	± 10.85
BDE-100	1µM	87.3*	± 1.15	95	± 5	96.8	± 8.1
	10µM	66.2*	± 1.04	79.5*	± 4.2	56.6*	± 7.39
BDE-153	1µM	77.5	± 9.89	98.6	± 4.77	72.9*	± 8.21
	10µM	42.1*	± 2.54	78.7*	± 1.37	40.2*	± 9.19
BDE-154	1µM	91.4	± 8.06	99.3	± 0.96	86.3	± 16.46
	10µM	85.7	± 6.54	91.3	± 7.57	70.9*	± 10.71
BDE-183	1µM	88.6	± 10.88	93.7	± 14.66	78.4	± 21.29
	10µM	46.2*	± 1.04	65.9*	± 13.26	39.9*	± 0.79

Table 2; Inhibitory effects of PBDEs on TCDD-induced EROD activity expressed as % efficacy compared to a maximal inducing concentration of TCDD (1 nM; 100 %). The data are expressed as mean ± standard deviation.*: significantly lower than the response to TCDD alone ($p<0.05$, $n=3$).

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CHAPTER THREE

Antagonism of TCDD-induced Ethoxyresorufin-O-deethylation activity by Polybrominated Diphenyl Ethers (PBDEs) in Primary Cynomolgus Monkey (*Macaca Fascicularis*) Hepatocytes.

A.K. Peters, J.T. Sanderson, Å. Bergman, M. Van den Berg
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Abstract

Polybrominated diphenyl ethers (PBDEs) are widespread environmental pollutants, and the levels of certain congeners have been increasing in biota and abiota in recent decades. Some PBDEs are lipophilic and persistent, resulting in bioaccumulation in the environment. Their structural similarity to other polyhalogenated aromatic hydrocarbons (PHAHs) such as polychlorinated biphenyls (PCBs) has raised concerns that PBDEs might act as agonists for the aryl hydrocarbon receptor (AhR). Recent studies in our laboratory with human and rat cell lines indicated no AhR mediated CYP1A1 induction for PBDEs. However, an earlier *in vitro* study by Van der Burght et al (*Toxicol Appl Pharmacol* (1999) 155, 13-23) indicated that in cynomolgus monkey (*Macaca fascicularis*) hepatocytes PCBs with a non-planar configuration could induce CYP1A. As PBDEs show a structural similarity with non-planar (*ortho* substituted) PCBs, our present study focused on the possible CYP1A induction by PBDEs (BDE-47, -99, -100, -153, -154, -183, and -77) in individual preparations (n=4) of primary hepatocytes of cynomolgus monkeys (*Macaca fascicularis*). 7-Ethoxyresorufin-O-deethylase (EROD) was used as a marker for CYP1A-mediated catalytic activity. Cells were exposed for 48 hours to various PBDE concentrations (0.01-10 µM), positive controls 2,3,7,8-TCDD (0.001-2.5 nM) and PCB-126 (0.01-10 nM), and negative control (DMSO vehicle alone).

No statistically significant induction of CYP1A was observed in the hepatocytes after 48 hours of exposure to all environmentally relevant PBDEs. After exposing hepatocytes to PBDEs in combination with TCDD, a concentration-dependent decrease in TCDD-induced EROD activity was observed. All PBDEs tested showed a similar reduction in each of four experiments, though quantitative differences were observed. The observed antagonism of TCDD-induced EROD activity by PBDEs occurred in both male (n=3) and female (n=1) hepatocytes and was not due to catalytic inhibition of EROD activity or cytotoxicity.

However, based on the results of this study we do not expect these antagonistic effects of PBDEs on CYP1A induction at environmentally relevant levels, since these *in vitro* interactive effects with TCDD were observed only at relatively high concentrations that are normally not seen e.g. in the human body.

Introduction

Brominated flame retardants (BFRs) make up approximately 40% of the worldwide flame-retardants market. BFRs can chemically reduce and retard the development of a fire because halogen atoms are released in its thermal decomposition. One group of BFRs, polybrominated diphenyl ethers (PBDEs), are used as additive flame-retardants at concentrations of 5-30% in plastic materials, paints, and textile fabrics. As a result of this large scale production and use, some PBDE congeners have been found in the environment, wildlife, and human population [1-6].

PBDEs are not acutely toxic and specific effects on human and environmental health are not well known. In laboratory studies PBDEs have been shown to interfere with the thyroid and estrogen hormone systems [7-9], or elicit neurobehavioral effects [10-13] and their human and environmental relevance should be further established.

The structural similarity of certain PBDE congeners to other polyhalogenated aromatic hydrocarbons (PHAHs), such as polychlorinated biphenyls (PCBs), has raised concerns that these compounds might activate aryl hydrocarbon receptor (AhR) mediated effects.

Dioxin-like compounds act as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which binds with high affinity to the AhR. Unbound AhR is present in the cytoplasm of the cell; after ligand binding, the complex translocates into the nucleus of the cell. The AhR dissociates in the nucleus and binds with a nuclear protein, ARNT (Ah receptor nuclear translocator). The AhR:ARNT complex then binds the dioxin responsive element (DRE) on the DNA, increasing the transcription of various genes, such as the *CYP1A1* gene [14,15]. Cytochrome P450 enzymes such as CYP1A1 are involved in the biotransformation of both endogenous and exogenous compounds. The activity and expression levels of these enzymes depends on genetic background, age, sex, diet and health status of the individual [16,17].

Previously, we investigated possible CYP1A1 induction by introducing highly-purified environmentally relevant PBDEs (BDE-47, -77, -99, -100, -153, -154, -183, -209) to human breast carcinoma (MCF-7), human hepatoma (HepG2), and rat hepatoma (H4IIE) cell lines [18] using 7-ethoxyresorufin-O-deethylation (EROD) activity as a marker for CYP1A1 activity [19]. We found no agonist activity, but we did measure an antagonistic or inhibitory effect after these cell lines were co-exposed to both TCDD and PBDEs in various concentrations. In our laboratory we have also found similar interactive effects between PBDEs and TCDD in primary carp hepatocytes (*Cyprinus carpio*) [20]. However, a weak but statistically significant

CYP1A1 induction has also been reported by BDE-77, -100, -153, and -183 in various mammalian cell lines [21,22] and in primary rat hepatocytes (*Rattus Norvegicus*) [21,23].
Clearly, primary cell cultures of hepatocytes provide a more physiologically relevant and possibly more sensitive system than cell lines, since they originate directly from body tissue [24-26]. Since the cynomolgus monkey is obviously a more closely related species to humans than rats and mice, it is clearly a more appropriate animal model to investigate the biological or toxicological effects of these compounds for the human situation. The molecular aspects of the metabolism of endogenous and exogenous compounds such as promutagens by CYP1A1 and CYP1A2 have been investigated; cynomolgus monkey hepatocytes do not constitutively express CYP1A1 and do not or very weakly constitutively express CYP1A2 [27-30]. However, CYP1A1 and CYP1A2 were upregulated after hepatocytes were exposed to inducers such as TCDD, certain PCBs, and 3-methylcholanthrene [27-29,31,32]. It has not yet been elucidated if the ethoxyresorufin is catalysed only by CYP1A1 or also by CYP1A2 in cynomolgus monkeys. For that reason, EROD activity in hepatocyte preparations from cynomolgus monkeys in our present study will be designated as CYP1A-mediated catalytic activity. In humans, CYP1A1 is not constitutively expressed in the liver but occurs in many extra-hepatic tissues; of the CYP1A-family only CYP1A2 has been identified in the human liver [33].

Results from an earlier *in vitro* study by Van der Burght et al. [27] with hepatocytes from cynomolgus monkeys and a wide range of PCB congeners indicated that CYP1A induction measured as EROD was not exclusively found for PCBs with more or less planar configurations e.g. congeners with no or one *ortho* chorine substitution. In this initial study, some PCB congeners with multiple *ortho* substitutions could also induce EROD activity although with a weak potency. Thus, with respect to the structure activity relationships (SAR) necessary to induce CYP1A activity by dioxin and dioxin-like compounds, the cynomolgus monkey might be different from rodent species. As from a structural point of view the environmentally relevant PBDEs most closely resemble non- planar *ortho* substituted PCBs, our present study focussed on the possibility of these brominated flame retardants to induce CYP1A activity.

In this study, we assessed the Ah receptor-mediated induction of CYP1A-mediated catalytic activity after exposure to highly purified environmentally relevant PBDEs (BDE-47, -99, -100, -153, -154, -183, and -77) in primary cell cultures of hepatocytes

of cynomolgus monkeys (*Macaca fascicularis*) (Table 1). In addition, we also examined if antagonistic or inhibitory effects of these PBDEs in combination with TCDD occur in hepatocytes of cynomolgus monkeys, in analogy as those observed in our earlier study with mammalian cell lines [18].

Structure of environmentally relevant PBDEs

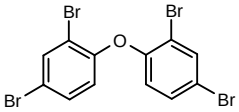
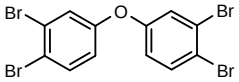
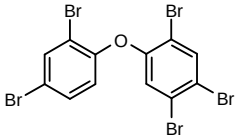
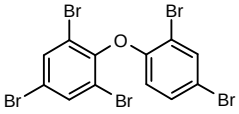
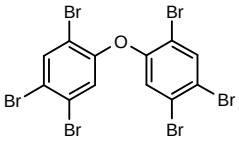
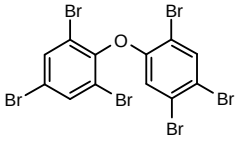
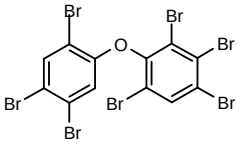
BDE-47	2,2',4,4'-tetrabromodipheyl ether		$C_{12}H_6Br_4O$
BDE-77	3,3',4,4'-tetrabromodiphenyl ether		$C_{12}H_6Br_4O$
BDE-99	2,2',4,4',5-pentabromodiphenyl ether		$C_{12}H_5Br_5O$
BDE-100	2,2',4,4',6-pentabromodiphenyl ether		$C_{12}H_5Br_5O$
BDE-153	2,2',4,4',5,5'-hexabromodiphenyl ether		$C_{12}H_4Br_6O$
BDE-154	2,2'4,4',5,6-hexabromodiphenyl ether		$C_{12}H_4Br_6O$
BDE-183	2,2',3,4,4',5',6-heptabromodiphenyl ether		$C_{12}H_3Br_7O$

Table 1; Environmentally relevant PBDEs used in this study: BDE-47, -99, -100, -153, -154, -183, -209 and BDE-77.

Materials and Methods

Chemicals

The chemicals used were obtained from the following companies: 2,3,7,8-TCDD (>99% pure) was purchased from Cambridge Isotope Laboratories (Woburn, MA, USA); 3,3',4,4',5-pentachlorobiphenyl (PCB-126) (>98% pure) was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany); PBDE congeners (>98% pure) were synthesized and each congener was subjected to a specific purification on activated charcoal and Celite to remove possible contamination with dioxin-like compounds such as PBDFs [34] (Table 1). The cell culture media (Williams E medium), Hanks' Balanced Salt Solution (HBSS), and fetal calf serum (FCS) were obtained from Gibco BRL (Breda, The Netherlands). Collagenase was obtained from Boehringer Ingelheim GmbH (Ingelheim, Germany). All other chemicals were obtained from Sigma Chemical Company (St. Louis, MO, USA).

Hepatocyte isolation

Male (n=3) and female (n=1) cynomolgus monkeys (*Macaca fascicularis*, 2-3 years old) were bred at the National Institute of Public Health and Environmental Protection (RIVM, Bilthoven, The Netherlands), where they served as donors for kidney cells used for the production of the poliomyelitis vaccine.

Following the nephrectomy, the liver was perfused *in situ* with phosphate buffered saline (PBS). After blood removal, the liver was removed and transported to our laboratory. The hepatocytes were isolated using the two-step collagenase perfusion technique described by Mennes *et al* [35].

The intact liver was perfused with Ca^{2+}- and Mg^{2+}-free HBSS containing 0.25 mM ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA) (500 ml, flow rate 80 ml/min), followed by a perfusion with Ca^{2+}- and Mg^{2+}-free HBSS (1000 ml, flow rate 80 ml/min). After these steps, collagenase was used to release the cells from the extracellular matrix. The cells were perfused with collagenase (0.5 mg/ml in HBSS) supplemented with 2.5 mM $CaCl_2$ and circulated for 30 min (flow rate 80 ml/min, pH 7).

Subsequently, the cells were shaken loose from the liver capsule in a 1% bovine serum albumin (BSA) solution in HBSS and incubated under a carbogen stream (10 min, 37ºC). The suspension was filtered, centrifuged (3 min, 600 rpm) and washed with the BSA solution. These steps were repeated several times until the supernatant was clear and only the hepatocytes were left.

Cell culture

The cells were seeded in 12-well plates (Greiner, Alphen a/d Rijn, The Netherlands) at a density of $0.7x10^6$ cells/well in Williams E medium supplemented with 1 µM insulin, 10 µM hydrocortisone, 5% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 4 mM $CaCl_2$ and 4 mM $MgCl_2$. After the first 4 hours, medium was replaced with medium without $CaCl_2$ and $MgCl_2$. After 12 hours the cells were exposed to environmentally relevant PBDEs (0.01-10 µM) and the positive controls TCDD (0.001-2.5 nM) and PCB-126 (0.01-10 nM) and negative control (DMSO) in serum-free medium without $CaCl_2$ and $MgCl_2$.

LDH assay

Plasma membrane integrity was determined by measuring lactate dehydrogenase (LDH) leakage into the culture medium after the cells were exposed to the test chemicals for 48h. One ml phosphate buffer with 66 mg/l pyruvate and 20 µl nicotinamide adenine dinucleotide (NADH) was added to 100 µl medium that was removed from the cells after the 48h incubation period. The reduction of NADH was measured spectrophotometrically at 340 nm. Controls were performed with 100 µl medium that was removed from cells that were not exposed to the test chemicals during the 48h incubation period and received 0.1% (w/v) Triton X-100 shortly prior to the LDH assay. This was set as 100% LDH release.

MTT assay

Cell viability, as an indicator for cytotoxicity, was determined by the capacity of cells to reduce MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma Chemical Company) to formazan [36]. This occurs in the presence of the mitochondrial enzymes succinate dehydrogenase. Disruption of mitochondrial function due to cytotoxicity will result in less formazan production. After exposure to the test chemicals for 48h, cells were washed with PBS and MTT (1 mg/ml in serum free Williams E medium) was added for 30 min at 37°C. The cells were washed with PBS and the formazan was extracted by adding 1 ml of isopropanol and incubating for 10 min. The formazan concentration was determined spectrophotometrically at an absorbance wavelength of 595 nm.

EROD assay

Ethoxyresorufin-O-deethylation (EROD) activity was used as a marker for CYP1A-mediated catalytic activity using a modification of the method described by Burke and Mayer (1974) as reported recently [18]. The cells were exposed to several

concentrations (0.01-10 µM) of PBDEs (Åke Bergman, Sweden), the positive controls TCDD (0.001-2.5 nM) and PCB-126 (0.01-10 nM), and a vehicle control (DMSO 0.1-0.2%). To determine antagonistic effects, cells were exposed to mixtures of TCDD (0.001-2.5 nM) and PBDEs (0.01-10 µM; solvent concentration ≤ 0.2%). After 48h, medium was removed and the cells washed twice with PBS (37°C). The intact cell monolayer was then exposed to 5 mM $MgCl_2$, 5 µM 7-ethoxyresorufin, and 10 µM dicumarol in 0.5 ml serum-free Williams E medium. The conversion of ethoxyresorufin to resorufin was followed using an excitation wavelength of 530 nm and emission wavelength of 590 nm (10 min, 37°C, Fluostar, BMG). The EROD activity was corrected for the amount of protein/ well [36].

Statistical analysis

In each experiment exposure to the test compound occurred in triplicate. EC_{50} values (50% of the maximum activity, calculated using the fitted concentration-response curve) were obtained using sigmoidal dose-response nonlinear regression curve fit (GraphPad Prism 3.0, GraphPad Software Inc., San Diego, CA). Statistical differences among treatments were determined by a two-tailed Student t-test, with a level of probability of 95% ($p<0.05$). The data are expressed as mean ± standard deviations, and one representative experiment is shown in the figures.

Results

A concentration-dependent increase of EROD activity was observed after a 48h incubation of primary cynomolgus monkey hepatocytes with the positive controls TCDD (0.001-2.5 nM) and PCB-126 (0.01-10 nM) (Figure 1). The relative induction potency of PCB-126 was one tenth (SD=0.04) of that of TCDD. The efficacy (maximal induction) of both compounds was approximately similar. None of the PBDEs caused a statistically significant increase of EROD activity. BDE-209 was initially included in this study, but the compound could not be tested within the chosen concentration range due to insolubility of the compounds at the chosen concentration range (≤ 10 µM, data not shown)

There were relatively large differences in the observed maximal EROD activity after 48h exposure to TCDD among the individual animals tested varying from 238±45.6 to 418±8.6 maximum absolute EROD activity; no difference was observed in maximal EROD activity between the female (n=1) and males (n=3). In three out of four individual experiments the exposure to 1 nM TCDD resulted in a maximal observed EROD activity and was set as 100% induction.

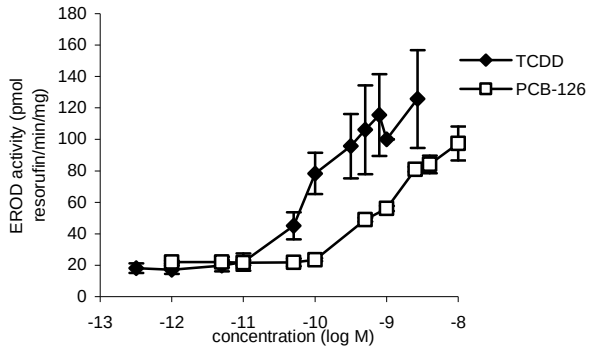


Figure 1; EROD activity in primary cell cultures of cynomolgus monkey (*Macaca fascicularis*) hepatocytes; exposed to positive controls TCDD (0.001-10 nM) and PCB-126 (0.01-10 nM). None of the PBDEs caused a statistically significant increase of EROD activity. Data are presented as mean (number of replicates =3) ± SD. Graphs show one representative experiment.

After co-exposure of the hepatocytes for 48h to TCDD and different PBDEs at various concentrations, a concentration-dependent decrease in TCDD-induced EROD activity was found. It was established that this decrease was due to antagonism of the AhR-regulated gene induction and not due to direct catalytic CYP1A inhibition by PBDEs, as no EROD inhibition was observed when TCDD-exposed hepatocytes (1 nM, 48h) were exposed to various PBDEs (10 µM) for 15 min prior to the EROD assay (data not shown).

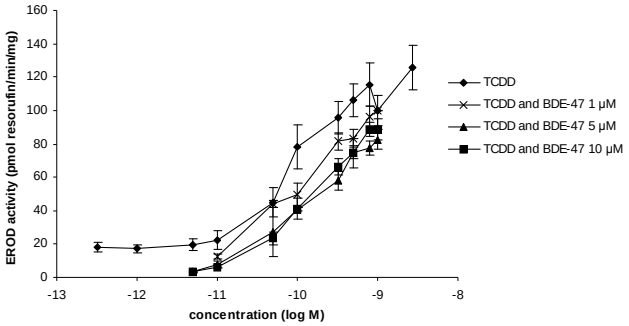
The observed antagonism by PBDEs on TCDD induced EROD activity is shown in figure 2, in which 1 nM of TCDD was set as maximal (100%) activity. It can be observed that this antagonism does not result in a significant shift in EC_{50} values of the co-exposed hepatocytes, but did result in a significant dose-dependent decrease in maximal inducible EROD activity (TCDD 1 nM) in the concentration range of 0.01 to 10 µM PBDE (Figure 2 (A-G)). This decrease in maximal induction of EROD activity compared to the positive control alone (TCDD 1 nM, 100%) is presented in table 2 in terms of percentage.

All tested PBDEs showed similar antagonistic effects, though not all were statistically significant. The effects varied between individual animals, with the more planar non-*ortho* substituted BDE-77 showing the strongest antagonism at 10 µM (Table 2).

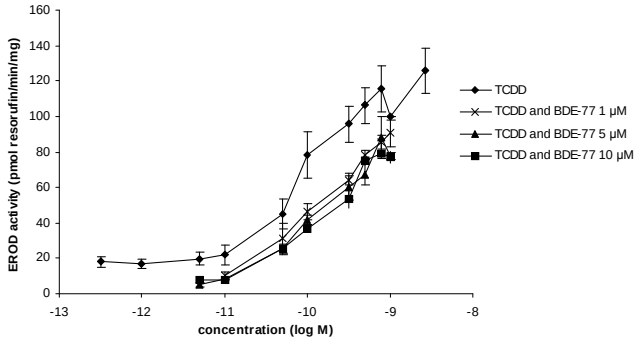
No effects on LDH leakage or formazan reduction were observed after a 48h exposure of the hepatocytes to PBDEs (0.01-10 µM) with and without co-exposure to the positive control TCDD (0.001-2.5 nM). The highest concentrations tested

were enforced by insolubility (PBDEs > 10µM) or cytotoxicity as measured by an increase in LDH leakage and a reduction of formazan (TCDD > 2.5 nM, PCB-126 > 10 µM) of the compounds (data not shown).

The viability of the hepatocytes was approximately 85-90% after the isolation procedure as observed with Trypan Blue (cell suspension was diluted with 0.6% Trypan blue) under a microscope (data not shown).



2A



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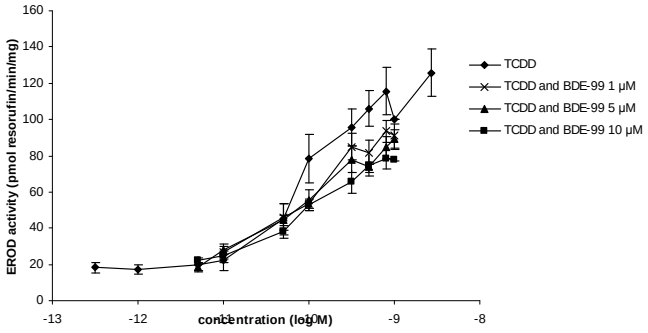

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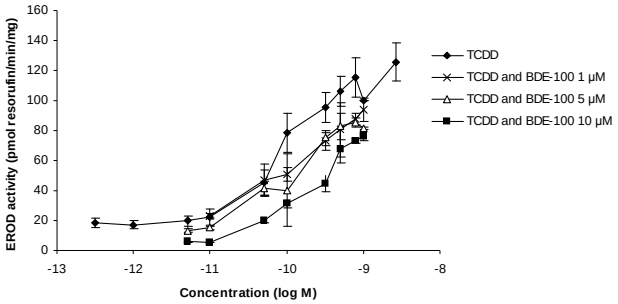
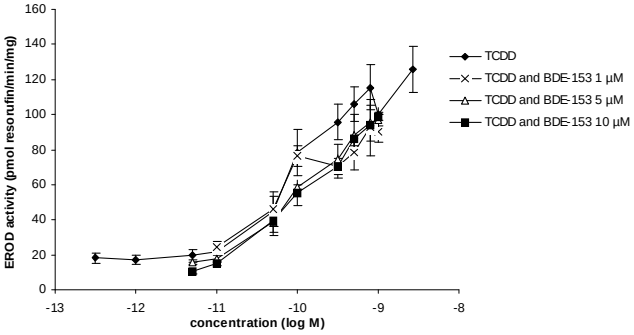
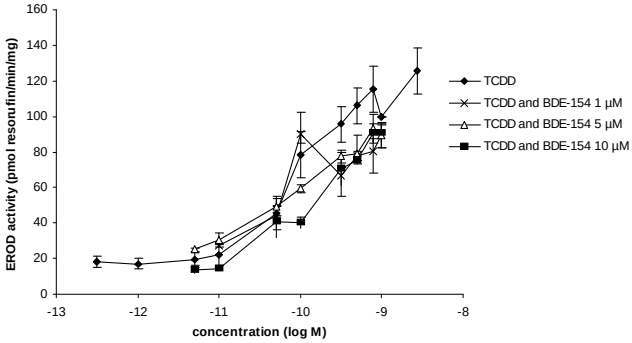

EROD activity (pmol resorufin/min/mg)
Concentration (log M)
TCDD
TCDD and BDE-100 1 µM
TCDD and BDE-100 5 µM
TCDD and BDE-100 10 µM

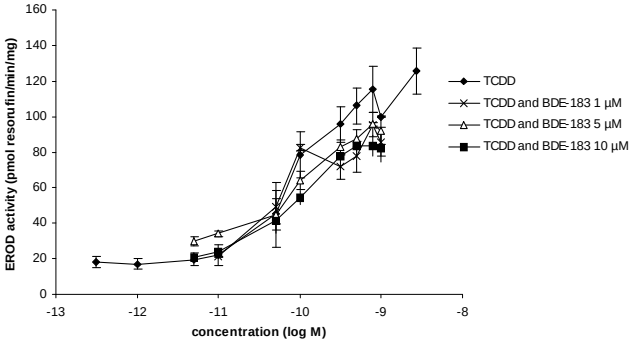
2D


EROD activity (pmol resorufin/min/mg)
concentration (log M)
TCDD
TCDD and BDE-153 1 µM
TCDD and BDE-153 5 µM
TCDD and BDE-153 10 µM

2E


EROD activity (pmol resorufin/min/mg)
concentration (log M)
TCDD
TCDD and BDE-154 1 µM
TCDD and BDE-154 5 µM
TCDD and BDE-154 10 µM

2F


EROD activity (pmol resorufin/min/mg)
concentration (log M)
TCDD
TCDD and BDE-183 1 µM
TCDD and BDE-183 5 µM
TCDD and BDE-183 10 µM

2G

Figure 2 (A-G); EROD activity in primary cell cultures of cynomolgus monkey (*Macaca fascicularis*) hepatocytes; co-exposed to TCDD and A) BDE-47 (1- 10 µM); B) BDE-77 (1- 10 µM); C) BDE-99 (1- 10 µM); D) BDE-100 (1- 10 µM); E) BDE-153 (1- 10 µM); F) BDE-154 (1-10 µM); G) BDE-183 (1- 10 µM). Data are presented as mean (number of replicates =3) ± SD. Graphs represent one representative experiment.

Discussion

In this study, we assessed the possible dioxin-like activity of environmentally relevant PBDE congeners BDE-47, -99, -100, -153, -154, -183, and -77 as expressed by the capability to induce CYP1A, measured as EROD activity in primary hepatocytes of cynomolgus monkey (Macaca fascicularis).

Our study was limited to three male and only one female cynomolgus monkey, thus we do not have any direct information about gender-differences, but no gender difference in cynomolgus monkeys was reported by Weaver et al [37]. Therefore, it can be suggested that the observed differences in the maximal inducibility in the hepatocytes by TCDD and efficacy of inhibition by PBDEs were not related to sex or age, but to inter-individual differences rather than by gender difference, which is in agreement with an earlier study [38].

Van der Burght et al demonstrated that several (multiple) *ortho*-substituted PCBs were able to induce CYP1A in hepatocytes from cynomolgus monkeys, although with weak potency [27]. The basis for our experiments was the assumption that PBDEs structurally resemble non-dioxin-like *ortho*-substituted PCBs, but nevertheless could induce CYP1A in the cynomolgus monkey. Previously, we demonstrated the inability of these PBDEs to induce EROD activity in human breast carcinoma (MCF-7), human hepatocellular carcinoma (HepG2), or rat hepatoma (H4IIE) cell lines [18]. In spite of the results from Van der Burght and co-workers (1999) in this *in vitro* model, no significant induction of EROD activity by these PBDEs was observed in cynomolgus monkey hepatocytes. Even the non-*ortho* substituted BDE-77 that structurally resembles the planar dioxin-like PCBs was not able to induce CYP1A in these hepatocytes. This lack of CYP1A induction of PBDEs in these monkey hepatocytes further supports the earlier conclusion [18,39] that PBDEs do not elicit AhR-mediated activity and therefore do not need to be assigned TEF values for risk assessment of dioxin-like compounds [40].

However, another study did find AhR activation by BDE-77, -100, -153, -183 in H4IIE cells or primary cell cultures of rat hepatocytes [21-23], although the observed

induction of CYP1A1 was small and occurred only at very high concentrations. In the human body, concentrations of PBDEs are currently maximally 7 ng/g lipid weight in Europe (breast milk) [1,13,41-44]. The observed dioxin-like induction of CYP1A1 activity occurs in the relatively high micromolar concentration range and seems to be at least a thousand-fold higher than current environmental levels in biota.

Primary cell cultures of hepatocytes are considered to be a more sensitive method to measure CYP1A(1) activity than cell lines [23-26]. However, this does not explain the discrepancy between the various studies in the observed or lack of induction of CYP1A by PBDEs. Based on the results of our previous study with mammalian cell lines, we suggested that a rigid and sensitive clean-up procedure is necessary to remove small amounts of AhR-active components to purify PBDEs properly [18]. Such contamination of the parent compounds with AhR components has been reported earlier for polychlorinated diphenyl ethers [45].

The lack of CYP1A induction of highly purified PBDEs in various *in vitro* rodent, monkey and human cell systems [18] most likely implies that there is no difference in interaction of PBDEs on the AhR signal transduction pathway between various species, assuming that CYP1A is one of the most sensitive markers for this type of interaction.

The PBDEs that were tested in our study all showed a concentration-dependent antagonistic effect towards TCDD-induced CYP1A activity (Figure 2 (A-G), Table 2). This antagonism was also observed in cell lines and primary hepatocytes of rat [18,23] and carp [20], but was not reflected in lower mRNA expression levels [18,23]. Cytotoxicity and direct catalytic inhibition were not observed in our present study, and can therefore be excluded as a reason for this antagonistic effect. Whether this antagonism by PBDEs on dioxin-like activity is caused by direct binding to the AhR, or interference with another part of the signal transduction pathway such as heme synthesis [46] cannot be concluded from our experiments and need to be studied in more detail. The fact that antagonism of PBDEs did not lead to a significant shift in EC_{50} values after co-exposure, but more clearly resulted in a decreased maximum in EROD activity, could originate from unproductive binding of these PBDEs to the AhR as suggested previously for PCB-153 [47]. This is currently under investigation in our laboratory with the use of stably transfected (AhR-EGFP) cell lines. Initial results suggest that the observed antagonism or inhibition of TCDD induced CYP1A1 might be a direct result of interaction and competition of these PBDEs with the AhR [39]. If this is the case, binding AhR by

PBDEs would not directly affect the response signal since AhR binding alone is required but not sufficient to elicit a response [48-50].
This would only have an implication on CYP1A mRNA and correlated CYP1A protein as measured in the EROD assay when all receptors are bound, e.g. at high concentrations of both the productive ligand (agonist) as well as the unproductive ligand (antagonist). It can be suggested that at lower concentrations of both the agonist as well as the antagonist, there would still be sufficient receptors available for the agonist that are able to activate the signal transduction pathway and elicit a response (i.e. CYP1A or EROD activity similar to that without an antagonist).

In conclusion, we have found no AhR-mediated induction of CYP1A activity by PBDEs that are relevant for humans and the environment in cynomolgus monkey hepatocytes. In addition, a dose-dependent antagonistic effect of PBDEs was observed on the induction of CYP1A, measured as EROD. These antagonistic effects were seen at PBDE concentrations ranging from 0.1 to 10 µM, while individual levels of PBDE in humans are estimated to be generally lower than 1 nM in the background situation in Europe [51]. Using experimental medium concentrations as a first approach for human plasma concentration a margin of several orders of magnitude seems apparent. In addition, this antagonistic effect was seen at relative high levels of induction of CYP1A by TCDD that are not easily expected in the human background population. Thus, the observed antagonistic effect of PBDEs on TCDD induced biological activity in our experiments probably does have little or no relevance for the human situation, but further (*in vivo*) studies may be necessary for confirmation.

Acknowledgements
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EROD activity in primary Cynomolgus Monkey hepatocytes

Compound	*EC50*				*Efficacy as % of maximal inducible by TCDD (1nM)*											
	♂	♂	♂	♀	♂			♂			♂			♀		
TCDD	$2.25*10^{-10}$	$6.61*10^{-11}$	$9.33*10^{-11}$	$9.77*10^{-11}$	100%			100%			100%			100%		
PCB-126	$2.67*10^{-9}$	$7.76*10^{-10}$	$1.15*10^{-9}$	$3.24*10^{-8}$												
TCDD+BDE-47 1 µM	$8.32*10^{-11}$	$7.94*10^{-11}$	$2.40*10^{-10}$	$5.75*10^{-11}$	89.2	± 2.7	*	88.5	± 11.7		99.7	± 9.0		84.6	± 14.9	
TCDD+BDE-47 5 µM	2.7610^{-10}	$7.94*10^{-11}$	$1.91*10^{-10}$	$1.11*10^{-10}$	90.1	± 2.7	*	86.8	± 10.1		82.6	± 5.9	*	66.2	± 13.4	
TCDD+BDE-47 10 µM	$1*10^{-10}$	$7.24*10^{-11}$	$1.41*10^{-10}$	$5.25*10^{-11}$	83.1	± 3.4	*	85.5	± 7.6	*	88.5	± 6.4	*	78.7	± 15.3	*
TCDD+BDE-77 1 µM	$8.71*10^{-11}$	$1.10*10^{-10}$	$1.41*1^{-10}$	$9.12*10^{-11}$	81.4	± 9.2	*	95.3	± 9.7		90.5	± 7.7		75.6	± 13.1	*
TCDD+BDE-77 5 µM	$8.13*10^{-11}$	$1.70*10^{-10}$	$1.15*10^{-9}$	$1.09*10^{-10}$	76.8	± 4.7	*	70.3	± 6.4	*	77.8	± 25.7	*	66.8	± 6.8	*
TCDD+BDE-77 10 µM	$9.33*10^{-11}$	$5.37*10^{-10}$	$9.33*10^{-11}$	$1.86*10^{-10}$	69.8	± 6.2	*	66.9	± 4.0	*	76.9	± 3.1	*	50.6	± 4.6	*
TCDD+BDE-99 1 µM	$9.55*10^{-11}$	$7.59*10^{-11}$	$2.40*10^{-10}$	$1.19*10^{-10}$	108.7	± 7.7		91.3	± 5.9		91.0	± 6.8		75.2	± 8.0	*
TCDD+BDE-99 5 µM	$7.59*10^{-11}$	$8.71*10^{-11}$	$2.40*10^{-10}$	$3.32*10^{-10}$	88.5	± 7.4	*	90.7	± 8.9		89.0	± 5.5	*	67.2	± 14.7	*
TCDD+BDE-99 10 µM	$8.71*10^{-11}$	$7.2*10^{-11}$	$1.41*10^{-10}$	$5.37*10^{-11}$	92.9	± 7.7		91.5	± 5.3	*	77.9	± 0.7	*	102.9	± 4.0	
TCDD+BDE-100 1 µM	$4.97*10^{-9}$	$1.10*10^{-10}$	$1.48*10^{-10}$	$1.69*10^{-10}$	98.9	± 6.1		91.7	± 7.0		93.7	± 7.8		91.8	± 26.6	
TCDD+BDE-100 5 µM	$7.59*10^{-11}$	$1.10*10^{-10}$	$1.48*10^{-10}$	$1.12*10^{-11}$	90.0	± 6.8		100.9	± 11.2		81.6	± 0.6	*	85.5	± 18.2	
TCDD+BDE-100 10 µM	ND	$6.17*10^{-9}$	$2.13*10^{-10}$	$8.32*10^{-11}$	84.8	± 3.0	*	105.1	± 10.9		78.0	± 75.9	*	88.5	± 7.5	
TCDD+BDE-153 1 µM	$9.12*10^{-11}$	$7.24*10^{-11}$	$1.74*10^{-10}$	$1.26*10^{-10}$	88.1	± 16.6		98.6	± 2.6		90.5	± 6.1	*	97.8	± 13.9	
TCDD+BDE-153 5 µM	$1.12*10^{-10}$	$7.94*10^{-11}$	$6.46*10^{-10}$	$4.37*10^{-11}$	83.3	± 10.8		92.1	± 6.9		96.6	± 4.6		92.1	± 4.3	*
TCDD+BDE-153 10 µM	6.9210^{-11}	$4.79*10^{-11}$	$8.51*10^{-11}$	$5.25*10^{-12}$	90.6	± 6.5		81.8	± 2.4	*	94.0	± 98.7		71.4	± 10.2	*
TCDD+BDE-154 1 µM	$9.12*10^{-11}$	$6.31*10^{-11}$	$5.6*10^{-11}$	$1.22*10^{-10}$	100.5	± 22.6		97.9	± 8.6		89.2	± 5.8	*	88.9	± 21.2	
TCDD+BDE-154 5 µM	$2.68*10^{-10}$	$4.79*10^{-11}$	$1.07*10^{-9}$	$9.55*10^{-11}$	115.9	± 14.8		91.9	± 7.6		89.2	± 7.1	*	87.1	± 6.0	*
TCDD+BDE-154 10 µM	$6.92*10^{-11}$	$7.08*10^{-11}$	ND	$2.63*10^{-11}$	109.6	± 12.4		98.2	± 12.1		90.8	± 5.3	*	72.9	± 18.5	
TCDD+BDE-183 1 µM	$1.73*10^{-10}$	$7.94*10^{-11}$	$5.13*10^{-11}$	$1.35*10^{-10}$	98.9	± 9.2		92.5	± 4.6	*	85.7	± 8.1	*	87.9	± 14.7	
TCDD+BDE-183 5 µM	$8.32*10^{-11}$	$8.91*10^{-11}$	$1.05*10^{-10}$	$7.76*10^{-11}$	85.7	± 6.5	*	99.4	± 3.0		92.2	± 7.9		92.4	± 3.0	*
TCDD+BDE-183 10 µM	$9.12*10^{-11}$	$7.41*10^{-11}$	$7.24*10^{-11}$	$8.13*10^{-11}$	90.1	± 6.7		74.5	± 0.9	*	82.6	± 8.1	*	82.5	± 16.8	

Table 2; EROD activity after co-exposure of primary cell cultures of cynomolgus monkey (*Macaca fascicularis*) hepatocytes to both TCDD (1 nM) and several PBDE congeners (1- 10 µM) for 48h. Data are presented as EC_{50} and efficacy (percentage of the maximal inducible concentration of TCDD (1 nM) ± SD).
*: statistically significant difference compared to TCDD 1 nM ($p<0.05$; number of replicates=3).

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CHAPTER FOUR

Interactions of Polybrominated Diphenyl Ethers (PBDEs) with the Aryl hydrocarbon receptor (AhR) pathway

A.K. Peters, S. Nijmeijer, K. Gradin, M. Backlund, Å. Bergman, L. Poellinger, M.S. Denison, M. Van den Berg

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Abstract

Polybrominated diphenyl ethers (PBDEs) are brominated flame retardants that have been in use as additives in various consumer products. Structural similarities of PBDEs with other polyhalogenated aromatic hydrocarbons that show affinity for the aryl hydrocarbon receptor (AhR), such as some polychlorinated biphenyls (PCBs), raised concerns about their possible dioxin-like properties. We studied the ability of environmental relevant PBDEs (BDE-47, -99, -100, -153, -154, and -183) and the relatively 'planar' congener BDE-77 to bind and/or activate the AhR in stably transfected rodent hepatoma cell lines with an AhR-responsive enhanced green fluorescent protein (AhR-EGFP) reporter gene (H1G1.1c3 mouse and H4G1.1c2 rat hepatoma). 7-Ethoxyresorufin-O-deethylation (EROD) was used as a marker for CYP1A1 activity. Dose- and bromination specific inhibition of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induced responses were measured by their ability to inhibit the induction of AhR-EGFP expression and EROD activity. Individual exposure to these PBDEs did not result in any increase in induction of AhR-EGFP or CYP1A1 activity. The lower brominated PBDEs showed the strongest inhibitory effect on TCDD induced activities in both cell lines. While the highest brominated PBDE tested, BDE-183, inhibited EROD activity, it did not affect the induction of AhR-EGFP expression. Similar findings were observed after exposing stably transfected human hepatoma (XRE-HepG2) cells to these PBDEs, resulting in a small but statically significant agonistic effect on XRE-driven luciferase activity. Co-exposure with TCDD again resulted in antagonistic effects, confirming that the inhibitory effect of these PBDEs on TCDD-induced responses was not only due to direct interaction at receptor level, but also at DNA binding level.

This antagonism was confirmed for BDE-99 in HepG2 cells transiently transfected with a Gal4-AhR construct and the corresponding Gal4-Luc reporter gene. In addition, a chromatin immunoprecipitation assay (ChIP) further confirmed that BDE-99 could bind to the AhR and activate the AhR nuclear translocation and DRE binding in the context of the CYP1A1 promoter. However, the transactivation function of the BDE-99 activated AhR seems to be very weak. These combined results suggest that PBDEs do bind, but do not activate the AhR-ARNT-XRE complex.

Introduction

Polybrominated diphenyl ethers (PBDEs) are a group of brominated flame retardants (BFR) and have been in use as additives in various consumer products since the 1970s. Although PBDEs are beneficial in that they reduce the chances of ignition and burning of materials, they have the ability to leach out of the polymer in which they are present. There are three major commercial PBDE mixtures that are or have been produced, differing in their degree of bromination; the Penta-, Octa- and Deca-mix. Some PBDE congeners have been found to be lipophilic with log Kow values varying from 6 for penta- to 10 for deca-BDE [1]. Accordingly, levels of PBDEs have been detected worldwide in many matrices e.g. in fish, wildlife, bird's eggs [2-5], as well as in human blood, adipose tissue [6,7] and breast milk samples [8].

The European Union Community banned the use of Penta-BDE and Octa-BDE since August of 2004[3], voluntarily followed by the sole US producer (Great Lakes Chemical Corporation, IN, USA) that ceased the production of these two PBDE mixtures by the end of 2004. The continued use of Deca-BDE is still under discussion within the United States, but its use is currently not restricted in the European Union[4]. The current ban on PBDEs will eventually lead to a decline in biota and abiota, as has already been reported in human breast milk samples from Sweden [9]. However, due to the persistent nature of PBDEs, it could take decades before this decline can be observed in all matrices worldwide with continuing exposure through older end-product use and waste incineration.

Structural similarities of PBDEs with other polyhalogenated aromatic hydrocarbons that bind to the aryl hydrocarbon receptor (AhR), such as some polychlorinated biphenyls (PCBs; Figure 1) raised concern about the possible binding of PBDEs to the AhR, leading to AhR-mediated toxic and biological effects. The AhR can be found in the cytoplasm of almost all vertebrate cells and a structurally diverse range of chemicals can bind to and/or activate AhR-dependent gene expressions, which leads to a variety of biological and toxic effects [10,11]. This ligand diversity suggests that the AhR has a rather promiscuous binding pocket

[3] European Union, Restriction of Hazardous Substances Directive (RoHS); Directive 2002/95/EC of the European Parliament and of the Council of 27 January 2003 on the restriction of the use of certain hazardous substances in electrical and electronic equipment, OJ L37, 13 February 2003, page 19

[4] Commission Decision of 13 October 2005 amending for the purposes of adapting to the technical progress the Annex to Directive 2002/95/EC of the European Parliament and of the Council on the restriction of the use of certain hazardous substances in electrical and electronic equipment, OJ L271, 15 October 2005, page 48

and raises questions as to the spectrum of chemicals which can bind to the AhR. Upon binding of a ligand to this receptor, the ligand:AhR complex migrates into the nucleus of the cell where it binds the AhR nuclear translocator protein (ARNT) [12]. The AhR and ARNT function together as a heterodimer and can bind to the xenobiotic response elements (XRE) on the DNA [13,14]. These XREs have been identified on the 5-prime promotor regions of several AhR inducible genes [15-17]. Binding of the ligand:AhR:ARNT complex to the XRE stimulates transcription of adjacent genes, the molecular mechanism of induction of expression of the phase I biotransformation enzyme cytochrome P4501A1 (CYP1A1) gene has been the most studied response [18].

CYP1A1 enhances the detoxification of lipophilic substrates (many of which are AhR ligands) that might otherwise accumulate to toxic concentrations in a cell. Induction of CYP1A1 only occurs when necessary; upon removal of the stimulus (ligand) by metabolism, CYP1A1 gene expression is no longer upregulated. TCDD is the highest affinity ligand for the AhR and it is also extremely slowly metabolized by CYP1A1 and other enzymes. Consequently, the long residence time of TCDD in the cell results in a sustained induction of gene expression and it is commonly thought that most of the potent toxic and biological effects of TCDD and related chemicals are attributed to the prolonged activation of the AhR-dependent gene expression [12]. CYP1A1 is inducible expressed in all vertebrates although there are large differences in expression levels in different tissues and among species [10,11,19]. Use of induction of CYP1A1 and CYP1A1-dependent ethoxyresorufin O-deethylase (EROD) activity as a biomarker for AhR-mediated effects in the risk assessment process developed for dioxin-like compounds has been proposed and applied [20].

Previously, we exposed rodent hepatoma (H4IIE), human breast carcinoma (MCF-7), and human hepatoma (HepG2) cell lines to highly-purified PBDEs (BDE-47, -99, -100, -153, -154, -183)[5] in order to assess their ability to activate the AhR mediated CYP1A1 induction using EROD activity as a marker. While these individual PBDEs were not able to induce CYP1A1, co-exposure resulted in a decrease in TCDD-induced CYP1A1 activity [22]. Similar results were observed for these PBDEs congeners using primary hepatocytes of cynomolgus monkeys (*Macaca fascicularis*) [23]. In contrast to our experiments, a weak but statistically significant CYP1A1 induction has been previously reported for the PBDEs -77, -100, -153, and -183 in various cell lines [24,25] and primary rat hepatocytes [24,26].

[5] The numbering of the PBDE congeners is adapted from the International Union of Pure and Applied Chemistry (IUPAC) system for numbering PCB congeners[21].

The objectives of our present study were to assess if the observed inhibitory effects of these PBDEs were caused by direct antagonism of the AhR and/or inhibition of the CYP1A1-dependent EROD activity. In addition to measuring CYP1A1 activity, PBDE interactions with the AhR were also assessed using two stably transfected rodent hepatoma cell lines containing an AhR-responsive enhanced green fluorescent protein (EGFP) reporter gene containing approximately 500 bp of the CYP1A1 promoter including 4 XRE sequences (H1G1.1c3 mouse and H4G1.1c2 rat hepatoma cell lines). A human hepatoma (HepG2) cell line containing either a stably transfected construct of two isolated XRE sequences in front of a heterologous promoter (XRE-HepG2) or transiently transfected with a Gal4-AhR construct, were used to investigate the AhR and XRE binding further.

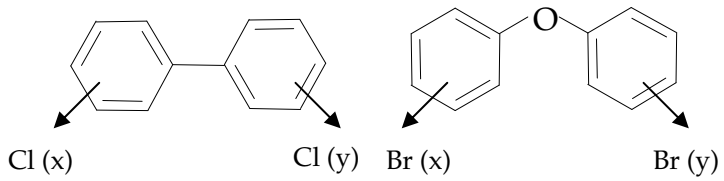


Figure 1; Structure of polychlorinated biphenyls (PCBs) (on the left) and polybrominated diphenyl ethers (PBDEs) (on the right), with (x+y ≤10)

Materials and Methods

Chemicals

The chemicals used were obtained from the following companies: 2,3,7,8-TCDD (>99% pure) was purchased from Cambridge Isotope Laboratories (Woburn, MA, USA); environmental relevant PBDE congeners (>98% pure) were synthesized and each congener was subjected to a specific purification on activated charcoal and Celite to remove possible contamination with dioxin-like compounds such as PBDFs [27]. Besides using the earlier mentioned environmental relevant PBDEs, BDE-77 was also included in our experiments. BDE-77 is not environmental relevant but resembles PCB-77 due to its lack of *ortho*-bromine and two adjacent bromine atoms on both aromatic rings, and was therefore included. H1G1.1c3 and H4G1.1c2 cell lines were provided by M.S. Denison (University of California-Davis, CA, USA), and XRE-HepG2 cells were provided by K. Gradin (University of Stockholm, Stockholm, Sweden); the HepG2 cell line was obtained from American

Type Culture Collection (ATCC, Manassa, VA, USA). Cell culture media Dulbecco's Modified Eagle Medium (DMEM), RPMI1640 with glutamine and phenol red, phosphate buffered saline (PBS), and fetal calf serum (FCS) were obtained from Gibco BRL (Breda, The Netherlands). Fugene 6 was purchased from Roche (Mannheim, Germany), Luciferase cell culture Lysis buffer (5x) reagent was purchased from Promega (Madison, WI, USA), luciferase buffer and ATP substrate were purchased from BioThema (Handen, Sweden). PCR reagents as well as OptiMEM were from Invitrogen (Carlsbad, CA, USA) and the protease inhibitor mix from Roche (Mannheim, Germany). All other chemicals were obtained from Sigma Chemical Company (St. Louis, MO, USA).

Cell culture

The mouse hepatoma cell line H1G1.1c3 and rat hepatoma cell line H4G1.1c2 were created by stable transfection of mouse hepatoma (Hepa1c1c7) and rat hepatoma (H4IIE) cells with the AhR-responsive Enhanced Green Fluorescent Protein (AhR-EGFP) reporter plasmid pGreen1 as reported earlier [28]. Both cell lines were cultured in DMEM supplemented with 10% heat inactivated FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin in an incubator (5% CO_2, 37ºC).

The HepG2-derived cell line XRE-HepG2 was created by stable transfection with a construct containing XRE sequences [29].The human hepatoma cell lines HepG2 and XRE-HepG2 were cultured in RPMI medium with phenol red and glutamine, supplemented with 10% heat inactivated FCS and 100 U/ml penicillin, and 100 µg/ml streptomycin. The XRE-HepG2 medium was additionally supplemented with 800 µg/ml geneticin.

Enhanced green fluorescent protein (EGFP) assay

The H1G1.1c3 and H4G1.1c2 cell lines have been genetically modified to produce a fluorescent protein upon activation of the AhR by ligands. Both cell lines were seeded in normal culture medium in 96 well plates ($1x10^4$ cells/well) and exposed after 24h to the solvent control DMSO (0.1%), positive control TCDD (0.001-1 nM), the chosen PBDEs (0.01-10 µM; Åke Bergman, Sweden), or co-exposed to both TCDD and PBDEs. After 72 hours the cells were washed twice with phosphate buffered saline (PBS, 37°C), PBS added to each well and the fluorescence of the intact cells was measured using an excitation wavelength of 485 nm and emission wavelength of 510 nm in a Fluostar (BMG). Dose response relationship analyses for the TCDD, PBDEs and TCDD/PBDEs co-exposures were carried out and induced

AhR-EGFP activity was determined by subtracting the background fluorescence in the DMSO sample from the fluorescence treated samples [30].

7-Ethoxyresorufin-O-deethylase (EROD) assay

Ethoxyresorufin-O-deethylation (EROD) activity was used as a marker for CYP1A1-mediated catalytic activity using a modification of the method described by Burke and Mayer (1974) as reported recently [22]. Directly following the AhR-EGFP assay, the PBS in the microplate wells was replaced with serum-free medium supplemented with 5 mM $MgCl_2$, 5 mM 7-Ethoxyresorufin (ER), and 10 mM dicumarol. The conversion of 7-ER into the fluorescent resorufin was followed over a 10 min period at 37ºC using an excitation wavelength of 530 nm and emission wavelength of 590 nm. The EROD activity was normalized to the amount of protein/well as measured by the method of Denziot and Lang (1986).

Transient transfection and luciferase assays

The Gal4-AhR construct and the corresponding Gal4-Luc reporter gene were created by cloning of the rat AhR into the mammalian expression vector pFA-CMV containing cDNA for the yeast Gal4 DNA binding domain, as described by Backlund et al [31]. Transient transfections into HepG2 cells were carried out in 24-well plates, using 150 ng of pFA-AhR expression plasmid and 200 ng of pFR-Luc per well in OptiMEM, as previously reported [31]. Fugene-6 transfection reagent was used according to the manufacturer's instructions (1.5 µl/well). Approximately 24h after transfection, the culture medium was exchanged with fresh media containing solvent control DMSO (0.1%), TCDD (0.001-1 nM), BDE-99 (0.01-10 µM), or a combination of TCDD and BDE-99. After 24h the transiently transfected cells or stable XRE-HepG2 cells were rinsed with PBS and 100-60 µl lysis buffer was added (12-24 well resp.). The cell lysates were collected by scraping and transferred to a microcentrifuge tube. After brief vortexing, cell lysates were spun (2-5 min, 4°C, maximum rpm), 30 µl of the supernatant was mixed with 100 µl Luciferin substrate and 100 µl ATP substrate and luciferase activity determined in a luminometer (Biotherm). The values were normalized to the protein concentrations measured according to the method of Bradford [32].

Chromatin Immunoprecipitation Assay (ChIP)

The procedure for the chromatin immunoprecipitation assay was adapted from that described by Brown and co-workers [33]. HepG2 cells were seeded in cell culture dishes (15 cm Ø) and grown until 80% confluency. The cells were then treated with either DMSO (0.1%), TCDD (1 nM), BDE-99 (10 µM), or TCDD and BDE-99 (1 nM and 10 µM respectively). After a three-hour incubation period, cells were washed with PBS. Protein cross-linking was achieved by adding formaldehyde (1%) to the cells and allowing them to incubate at room temperature for 10 minutes. Cells were washed twice with ice-cold PBS, collected into a 100 mM Tris-HCL (pH 8.7) and 10 mM dithiothreitol (DTT) solution (cold) into a centrifuge tube, incubated for 15 min at 30°C, and subsequently pelleted (5 min, 2000g). The pellets were washed sequentially in the following buffers: PBS (ice-cold); buffer I (0.25% TritonX-100, 10 mM EDTA, 0.5 mM EGTA, 10 mM HEPES (pH 6.5)); buffer II (200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM HEPES (pH 6.5)). The pellets were resuspended in 0.5 ml lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.0), and protease inhibitor cocktail) and then sonified on ice (three times for 10 sec, Branson Sonifier) to yield DNA fragments in the 200 to 900 bp range. Samples were centrifuged for 10 min at 4°C, and the supernatants were mixed (1:10) with dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl (pH 8.0), protease inhibitor cocktail). IgG immunoclearing was performed by incubating 1 ml soluble chromatin with 2 µg sheared herring sperm DNA, 6 µg IgG, and 45 µl protein G-sepharose (50% slurry) for 2h at 4°C with rotation. After centrifugation (15 sec, 3000 rpm), the supernatant was incubated overnight with 5 µg of AhR-specific antibodies or IgG (overnight at 4°C with rotation). After this, 45 µl of protein G-Sepharose slurry containing 2 µg sheared herring sperm DNA was added to the samples (2h at 4°C rotation). The sepharose beads were collected by centrifugation (15 sec, 3000 rpm) and washed sequentially in the following buffers (15 min rotation): TSE I (20 mM Tris-HCL (pH 8.1), 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100); TSE II (20 mM Tris-HCL (pH 8.1), 500 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100), buffer III (10mM Tris-HCL (pH 8.1), 0.5 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA) and twice with TE-buffer. Immunocomplexes were extracted three times from the beads with 100 µl 1% SDS-0.1 M $NaHCO_3$ and centrifuged at 3000 rpm for 15 sec.

Cross-linking was reversed by heating the eluates at 65°C overnight. The eluates were then purified with QIAquick Spin Kit and 1 µl of the purified DNA (0.1 µl for the input sample) was used for PCR (Platinum *Pfx* DNA Polymerase; performed according to the manufacturer's recommendations) with the primers that amplify

regions containing the XRE elements of the human *CYP1A1* gene (hCYP1A1-XRE 5' -CAC CCT TCG ACA GTT CCT CTC CCT and hCYP1A1-XRE 3' -CTC CCG GGG TGG CTA GTG CTT TGA). The PCR products were separated and visualized on a 2% agarose gel.

Cell viability

After incubation with the designated compounds for 72h, cells were washed and medium was replaced with a 1 mg/ml MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, Sigma Chemical Company) solution. The formation of MTT to formazan in the presence of the mitochondrial enzymes succinate dehydrogenase was allowed to proceed for 30 min at 37°C [34]. After the incubation period, cells were washed and the formazan was extracted with 0.1 ml of isopropanol and incubated for 10 min. The formazan concentration was determined spectrophotometrically using an absorbance wavelength of 595 nm.

Statistical analysis

In each experiment exposure to the test compound was carried out in triplicate. EC_{50} values for EROD activity and AhR-EGFP response (50% of the maximum activity, calculated using the fitted concentration-response curve) were obtained using sigmoidal dose-response nonlinear regression curve fit (GraphPad Prism 3.0, GraphPad Software Inc., San Diego, CA). Statistical differences among treatments were determined by a two-tailed Student t-test, with a significance level at >95% ($p<0.05$).

Results

Exposure of mouse H1G1.1c3 and rat H4G1.1c2 cells to the positive control agonist TCDD resulted in a concentration-, dose- and time-dependent increase in TCDD-induced CYP1A1 (EROD) activity and AhR-EGFP reporter gene expression. No difference in sensitivity in induction between the H1G1.1c3 and H4G1.1c2 cell lines was observed with the EROD assay (Figure 2A), with EC_{50} values for TCDD of $8.18x10^{-12}$ M and $4.67x10^{-12}$ M, respectively. However, with respect to AhR-EGFP induction (Figure 2B), the mouse hepatoma cell line was more sensitive, with EC_{50} values of $4.03x10^{-12}$ M for H1G1.1c3 cells and $9.16x10^{-12}$ M for H4G1.1c2 cells. These almost similar EC_{50} values were caused by the fact that the mouse H1G1.1c3 cell line reached a higher maximum induction (Figure 2B). Although these cell lines are exquisitely sensitive bioassays to detect inducers of AhR-dependent gene

expression, no induction of EGFP or CYP1A1/EROD was observed for any individual PBDE (data not shown).

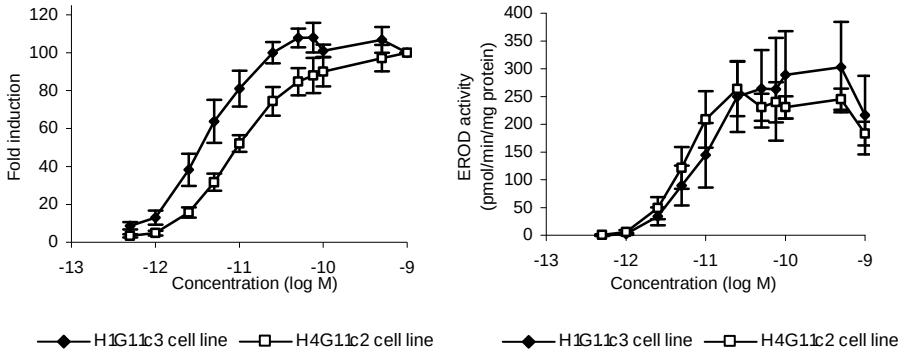
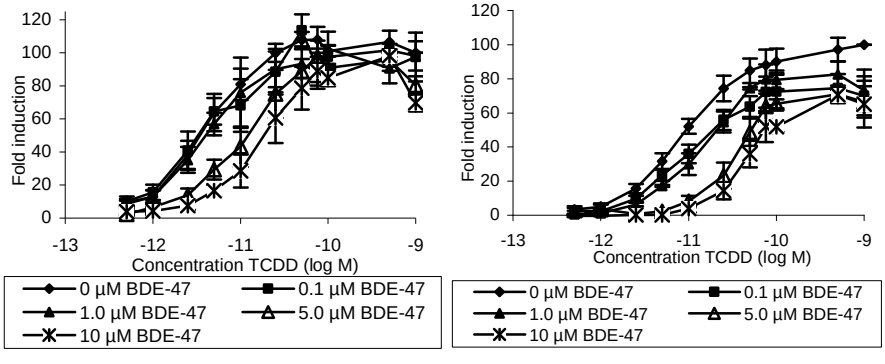


Figure 2; Induction of AhR-EGFP expression (on the left) and EROD-activity (on the right) in mouse H1G1.1c3 cells and rat H4G1.1c2, after exposure to TCDD. The data are expressed as mean of three separate experiments ± SEM.

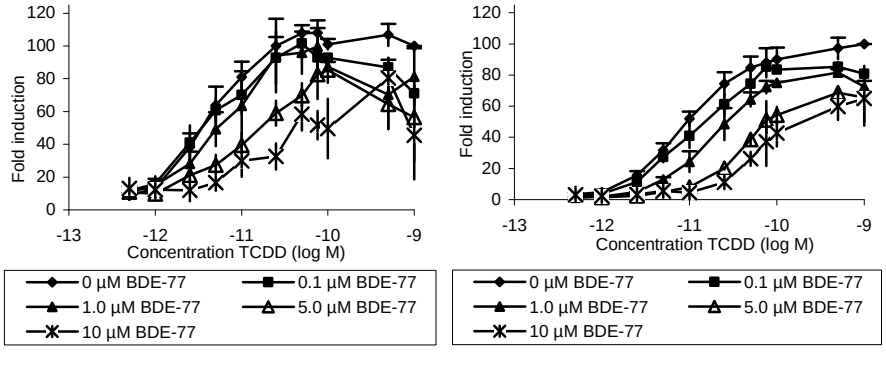
Antagonistic effects of PBDEs on AhR-EFGP expression

Co-incubation of TCDD (0.001 to 1nM) with 0.1-10 µM PBDEs (BDE-47, -99, -100, -153, -154, -183 and -77) resulted in a concentration-dependent decrease in AhR-EGFP expression (Figure 3A-C). This antagonistic effect was observed for most PBDEs tested, although the non-*ortho* substituted BDE-77 exhibited the greatest antagonistic effect in both cell lines (Figure 3B). A quantitative difference in antagonistic effects was observed between the PBDEs tested, which appeared related to their degree of bromination (Table 1, 2). Lower brominated PBDEs such as the tetrabrominated BDE-47 and -77, showed stronger antagonism of TCDD induced AhR-EGFP activity compared to higher brominated PBDEs, while the highest brominated congener tested (heptabrominated BDE-183) failed to antagonize AhR-EGFP reporter gene induction (Figure 3C).

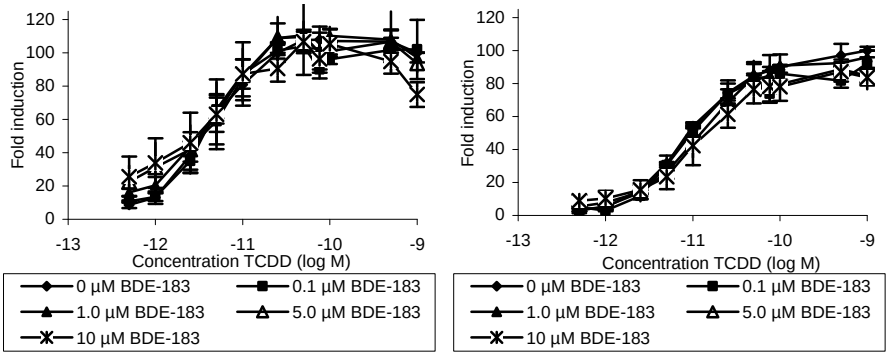
No striking differences were observed between the two cell lines, though the AhR-EGFP expression resulted in more significant effects in the rat hepatoma cell line (Table 1, 2).



3A

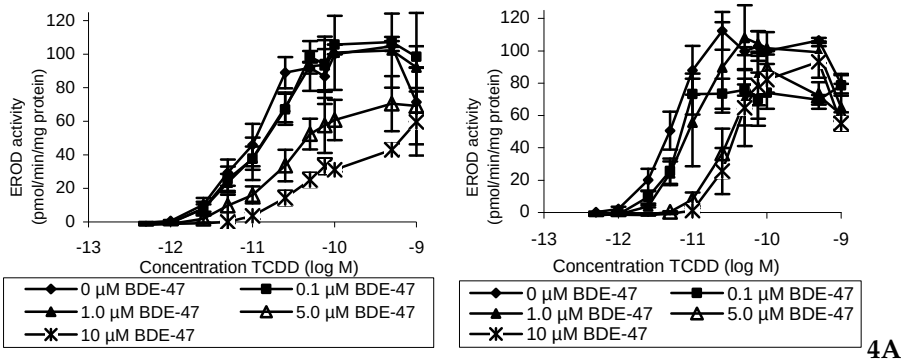


3B

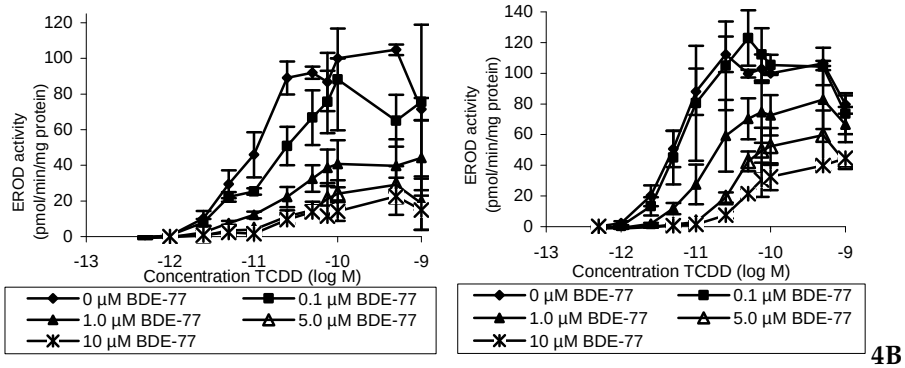


3C

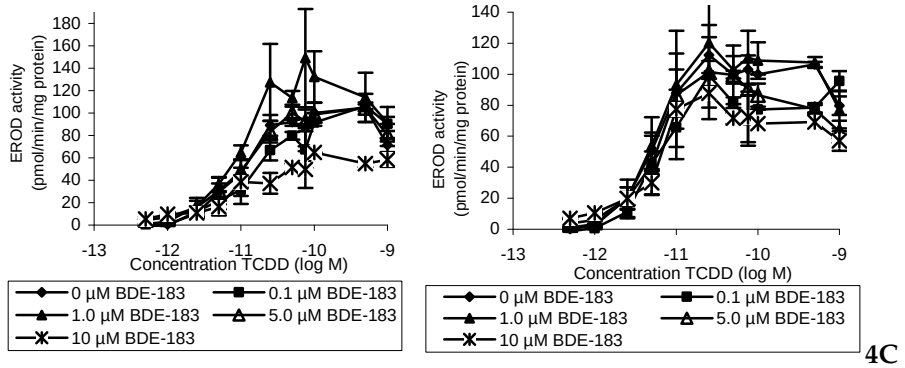
Figure 3; Induction of AhR-EGFP expression in mouse H1G1.1c3 (on the left) and rat H4G1.1c2 (on the right) cells after co-exposure to TCDD (0.5 pM-1 nM) and (A) BDE-47 (0-10 µM), (B) BDE-77 (0-10 µM), (C) BDE-183 (0-10 µM). The data are expressed as mean of three separate experiments ± SEM.



4A



4B



4C

Figure 4; Induction of EROD activity in mouse H1G1.1c3 (on the left) and rat H4G1.1c2 (on the right) cells after co-exposure to TCDD (0.5 pM-1 nM) and (A) BDE-47 (0-10 µM), (B) BDE-77 (0-10 µM), (C) BDE-183 (0-10 µM). The data are expressed as mean of three separate experiments ± SEM.

Antagonistic effects of PBDEs on EROD activity

Consistent with the above results, a concentration-dependent decrease in EROD activity was also observed (Figure 4). These effects occurred at higher concentrations than measured in the AhR-EGFP assay, resulting in lower EC_{50} values (Table 1, 2). As with the AhR-EGFP assay, this antagonistic/inhibitory effect was more pronounced with the lower brominated PBDEs, with the non-*ortho* substituted BDE-77 being the most potent antagonist in both cell lines (Figure 4B) and BDE-183 hardly showing any effect. No obvious differences were observed between the two cell lines, though the mouse hepatoma cell line showed a more pronounced effect in the EROD assay as can be seen in table 1 and table 2.

As with the AhR-EGFP assay, this effect was more pronounced with lower brominated PBDEs, with the non-*ortho* substituted BDE-77 as strongest antagonist in both cell lines (Figure 4B).

No obvious differences were observed between the two cell lines, though the mouse hepatoma cell line showed a more pronounced effect in the EROD assay as can be seen in table 1 and table 2.

Transfections in HepG2 cells

The human hepatoma cell line (HepG2) containing a stably transfected XRE-driven luciferase-reporter gene was also used to study the possible AhR-mediated dose- and time-dependent activation of transcription by different PBDEs. Exposure of the cells to the positive control TCDD resulted in a dose-dependent significant induction (EC_{50} $6.7x10^{-10}$ M). The PBDEs resulted in a small though not always significant induction of XRE-driven luciferase activity (at most 2% of that of TCDD 1 nM for BDE-99 10 µM) compared to the solvent control. The non-*ortho* substituted BDE-77 that proved the most potent antagonist in the EROD and AhR-EGFP assays did not result in a remarkably higher induction of XRE-driven luciferase activity compared to other lower brominated congeners as BDE-47 and BDE-99 (data not shown). BDE-99 resulted in the most consistent significant luciferase activity.

Table 1 (page 80); Effects of PBDEs on TCDD-induced AhR-EGFP expression and EROD activity in a stably transfected mouse hepatoma cell line (H1G1.1c3). The data are presented as the mean of three experiments (± SEM), all conducted in triplicate. *: statistically significant ($p<0.05$)

Table 2 (page 81); Effects of PBDEs on TCDD-induced AhR-EGFP expression and EROD activity in a stably transfected rat hepatoma cell line (H4G1.1c2). The data are presented as the mean of three experiments (± SEM), all conducted in triplicate. *: statistically significant ($p<0.05$)

Table 1

Effect of PBDEs on TCDD-induced AhR-EGFP expression and EROD activity in mouse H1G1.c1 cells

	Concentration (nM)	EC50	EROD	t-Test			Concentration (nM)	EC50	CAFLUX	t-Test	
TCDD	0.1	$8.18*10^{-12}$	100			TCDD	1	$4.03*10^{-12}$	100	*p*	
	Concentration (µM)		Y-max	*p*			Concentration (µM)		Y-max		
BDE-47	0.1	$1.45*10^{-11}$	105.8 ± 39	0.89		BDE-47	0.1	$3.62*10^{-12}$	98.2 ± 9	0.85	
	1	$1.10*10^{-11}$	100.9 ± 22	0.97			1	$4.07*10^{-12}$	97.5 ± 15	0.88	
	5	$1.86*10^{-11}$	60.8 ± 12	0.03	*		5	$1.02*10^{-11}$	80.3 ± 5	0.02	*
	10	$4.65*10^{-11}$	31.1 ± 1	0.00	*		10	$1.57*10^{-11}$	69.7 ± 5	0.00	*
BDE-77	0.1	$1.18*10^{-11}$	88.2 ± 28	0.70		BDE-77	0.1	$3.49*10^{-12}$	71.0 ± 5	0.00	*
	1	$1.75*10^{-11}$	40.8 ± 13	0.01	*		1	$5.33*10^{-12}$	81.3 ± 17	0.34	
	5	$2.62*10^{-11}$	24.1 ± 8	0.00	*		5	$1.01*10^{-11}$	56.4 ± 27	0.18	
	10	$2.02*10^{-11}$	14.1 ± 5	0.00	*		10	$2.14*10^{-11}$	45.5 ± 27	0.11	
BDE-99	0.1	$8.89*10^{-12}$	107.8 ± 31	0.81		BDE-99	0.1	$4.33*10^{-12}$	95.2 ± 25	0.85	
	1	$9.30*10^{-12}$	96.7 ±5	0.55			1	$4.68*10^{-12}$	102.2 ± 7	0.77	
	5	$1.49*10^{-11}$	56.0 ± 11	0.02	*		5	$7.54*10^{-12}$	88.9 ± 18	0.56	
	10	$2.97*10^{-11}$	47.1 ± 5	0.00	*		10	$1.08*10^{-11}$	83.8 ± 16	0.36	
BDE-100	0.1	$7.13*10^{-12}$	130.7 ± 5	0.00	*	BDE-100	0.1	$3.39*10^{-12}$	73.6 ± 4	0.00	*
	1	$7.91*10^{-12}$	100.1 ± 10	0.99			1	$3.83*10^{-12}$	94.7 ± 13	0.70	
	5	$6.99*10^{-12}$	97.4 ± 15	0.87			5	$5.39*10^{-12}$	74.7 ± 15	0.17	
	10	$8.92*10^{-12}$	63.6 ± 10	0.02	*		10	$6.69*10^{-12}$	87.2 ± 18	0.52	
BDE-153	0.1	$7.37*10^{-12}$	116.7 ± 13	0.28		BDE-153	0.1	$3.57*10^{-12}$	90.6 ± 8	0.29	
	1	$7.16*10^{-12}$	97.7 ± 7	0.74			1	$3.80*10^{-12}$	96.0 ± 7	0.61	
	5	$7.67*10^{-12}$	66.5 ± 10	0.03	*		5	$5.04*10^{-12}$	96.2 ± 15	0.82	
	10	$8.72*10^{-12}$	63.3 ± 9	0.01	*		10	$4.90*10^{-12}$	93.4 ± 9	0.52	
BDE-154	0.1	$7.81*10^{-12}$	148.4 ± 15	0.03	*	BDE-154	0.1	$3.95*10^{-12}$	93.5 ± 18	0.81	
	1	$6.23*10^{-12}$	125.0 ± 20	0.28			1	$3.43*10^{-12}$	80.4 ± 3	0.00	*
	5	$7.82*10^{-12}$	101.9 ± 20	0.93			5	$5.00*10^{-12}$	76.8 ± 11	0.22	
	10	$5.21*10^{-12}$	50.1 ± 4	0.00	*		10	$7.38*10^{-12}$	88.1 ± 6	0.11	
BDE-183	0.1	$1.16*10^{-11}$	91.4 ± 33	0.81		BDE-183	0.1	$3.91*10^{-12}$	102.0 ± 4	0.62	
	1	$7.77*10^{-12}$	132.2 ± 23	0.23			1	$4.73*10^{-12}$	97.2 ± 14	0.85	
	5	$8.31*10^{-12}$	98.8 ± 10	0.91			5	$5.00*10^{-12}$	93.7 ± 5	0.29	
	10	$9.97*10^{-12}$	64.8 ± 35	0.37			10	$4.40*10^{-12}$	74.9 ± 7	0.03	*

Effect of PBDEs on TCDD-induced AhR-EGFP expression and EROD activity in rat H4G1.c1 cells

	Concentration	EC50	EROD	t-Test			Concentration	EC50	CAFLUX	t-Test	
	(nM)						(nM)				
TCDD	0.1	$4.67*10^{-12}$	100	*p*		TCDD	1	$9.16*10^{-12}$	100	*p*	
	Concentration		Y-max				Concentration		Y-max		
	(µM)						(µM)				
BDE-47	0.1	$5.45*10^{-12}$	74.1 ± 8	0.03	*	BDE-47	0.1	$1.01*10^{-11}$	70.0 ± 11	0.06	
	1	$7.95*10^{-12}$	101.7 ± 10	0.87			1	$1.38*10^{-11}$	73.2 ± 12	0.09	
	5	$2.51*10^{-11}$	88.7 ± 9	0.28			5	$3.41*10^{-11}$	66.5 ± 9	0.02	*
	10	$2.96*10^{-11}$	82.2 ± 18	0.38			10	$4.69*10^{-11}$	65.2 ± 14	0.06	
BDE-77	0.1	$5.43*10^{-12}$	105.5 ± 7	0.45		BDE-77	0.1	$1.05*10^{-11}$	80.8 ± 5	0.02	*
	1	$1.23*10^{-11}$	72.7 ± 13	0.11			1	$1.76*10^{-11}$	72.5 ± 4	0.00	*
	5	$3.00*10^{-11}$	52.3 ± 12	0.02	*		5	$4.38*10^{-11}$	65.6 ± 15	0.09	
	10	$4.84*10^{-11}$	32.6 ± 9	0.00	*		10	$6.75*10^{-11}$	65.0 ± 17	0.12	
BDE-99	0.1	$5.60*10^{-12}$	72.7 ± 1	0.00	*	BDE-99	0.1	$8.96*10^{-12}$	87.5 ± 14	0.41	
	1	$6.66*10^{-12}$	101.7 ± 15	0.91			1	$9.97*10^{-12}$	89.0 ± 11	0.39	
	5	$1.03*10^{-11}$	92.1 ± 16	0.65			5	$1.71*10^{-11}$	80.0 ± 3	0.00	*
	10	$1.49*10^{-11}$	115.7 ± 36	0.68			10	$2.41*10^{-11}$	77.8 ± 13	0.18	
BDE-100	0.1	$5.09*10^{-12}$	173.8 ± 64	0.32		BDE-100	0.1	$7.58*10^{-12}$	89.8 ± 10	0.37	
	1	$5.25*10^{-12}$	140.5 ± 44	0.41			1	$7.55*10^{-12}$	82.7 ± 7	0.08	
	5	$6.85*10^{-12}$	127.9 ± 37	0.49			5	$9.95*10^{-12}$	81.0 ± 9	0.11	
	10	$1.00*10^{-11}$	88.2 ± 18	0.56			10	$1.21*10^{-11}$	60.2 ± 4	0.00	*
BDE-153	0.1	$5.56*10^{-12}$	79.7 ± 2	0.00	*	BDE-153	0.1	$7.57*10^{-12}$	79.8 ± 11	0.14	
	1	$4.60*10^{-12}$	108.3 ± 9	0.41			1	$7.19*10^{-12}$	78.4 ± 15	0.22	
	5	$3.83*10^{-12}$	94.9 ± 5	0.39			5	$7.01*10^{-12}$	71.5 ± 5	0.01	*
	10	$3.63*10^{-12}$	90.0 ± 13	0.48			10	$7.89*10^{-12}$	77.3 ± 9	0.07	
BDE-154	0.1	$4.53*10^{-12}$	129.8 ± 15	0.11		BDE-154	0.1	$7.46*10^{-12}$	82.0 ± 4	0.01	*
	1	$3.96*10^{-12}$	115.1 ± 12	0.29			1	$7.58*10^{-12}$	83.1 ± 6	0.06	
	5	$4.11*10^{-12}$	89.9 ± 12	0.45			5	$7.37*10^{-12}$	75.5 ± 9	0.05	*
	10	$4.08*10^{-12}$	64.1 ± 13	0.05	*		10	$1.02*10^{-11}$	67.8 ± 13	0.07	
BDE-183	0.1	$4.87*10^{-12}$	77.2 ± 12	0.14		BDE-183	0.1	$7.86*10^{-12}$	92.1 ± 2	0.02	*
	1	$4.71*10^{-12}$	108.8 ± 12	0.50			1	$8.46*10^{-12}$	95.9 ± 8	0.62	
	5	$5.15*10^{-12}$	86.6 ± 7	0.15			5	$1.03*10^{-11}$	84.6 ± 8	0.13	
	10	$5.99*10^{-12}$	68.2 ± 26	0.28			10	$1.26*10^{-11}$	83.6 ± 5	0.03	*

After co-incubation of the XRE-HepG2 cells with TCDD and selected PBDEs, the human cell line responded similar to the rodent cell lines. All PBDEs caused an antagonistic effect towards the TCDD-induced XRE induction. The AhR-EGFP construct contains 500 bp of the CYP1A1 promotor and the HepG2-XRE construct contains isolated XRE sequences; the fact that the PBDEs are able to inhibit TCDD-induced responses in both systems is an indication that the inhibition is mediated via the XRE sequences.

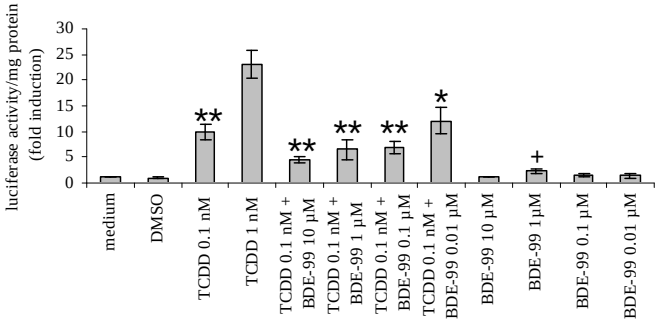


Figure 5; Luciferase activity in HepG2 cells transiently transfected with Gal4-AhR expression vector and the corresponding Gal4-Luc reporter gene. Cells were exposed to solvent control DMSO (0.1%), TCDD (1 nM), BDE-99 (0.01-10 µM), and a combination of TCDD (1 nM) and BDE-99 (0.01-10 µM). Data are expressed as mean of one representative assay, ± SEM (n=3, *; $p < 0.05$ compared to TCDD (1 nM), **; $p < 0.01$ compared to TCDD (1 nM), +; $p < 0.05$ compared to DMSO)

Since the most consistent results in the XRE-HepG2 luciferase assay were obtained with BDE-99, HepG2 cells were transiently transfected with a Gal4-AhR construct and the corresponding Gal4-Luc reporter gene and exposed to BDE-99, with and without co-exposure to the TCDD. Exposure to TCDD resulted in a dose-dependent increase in luciferase activity in this cell line (EC_{50} $3.7x10^{-10}$ M). However, exposure to BDE-99 (0.01-10 µM) alone did not result in a significant induction of AhR expression (Figure 5). The Gal4-AhR expression is driven by the CMV-promotor, activity of the co-transfected Gal4-dependent luciferase reporter gene is only detected after stimulation of the cells with AhR activators. Dimerization of the AhR/ARNT complex with the DNA is not needed to obtain a response in cells transiently transfected with this construct. Thus, BDE-99 by itself did not activate the Ah receptor-XRE complex. Co-exposure of the cells with TCDD and BDE-99 again resulted in a dose-dependent decrease in AhR expression, suggesting a similar type of interaction as observed in the AhR-GFP cell lines.

Chromatin Immunoprecipitation Assay (ChIP)

To further assess at what point BDE-99 was able to interfere with the signal transduction pathway, a ChIP assay was performed with cells treated with DMSO (0.1%), TCDD (1 nM), BDE-99 (10 µM), or TCDD and BDE-99 (1 nM and 10 µM respectively). The AhR antibody resulted in a positive band for both BDE-99 and the co-exposed cells, but the brightest band appeared with TCDD exposed cells (Figure 6). This supports the data obtained in the AhR-EGFP and transfection assays, further suggesting some PBDEs can bind to and stimulate AhR DNA binding, but that the interaction with DNA and chromatin leads to an unproductive complex incapable of activating transcription of the *CYP1A1* gene. While similar types of antagonism have been observed for some steroid hormone nuclear receptors, this would be the first AhR antagonist that exerts its inhibitory effects in this manner. The control antibody IgG did not result in any unexpected bands on the gel (Figure 6).

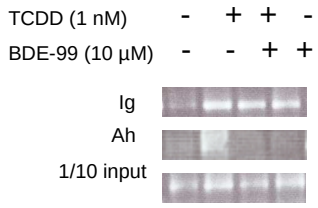


Figure 6; End products of the Chromatin Immunoprecipitation Assay (ChIP) on a 2% agarose gel.

Discussion

PBDEs have been suggested to act as AhR antagonists based on their ability to inhibit TCDD-induced CYP1A1 activity in human and rodent cell lines [22,23]. In the present study, we determined whether these PBDEs could antagonize TCDD-induced AhR-mediated effects either by direct effect on binding and/or activation of the AhR.

In both AhR-EGFP rodent cell lines, exposure to the PBDEs alone did not result in any AhR-mediated signals. As could be expected from a mechanistic point of view this also resulted in a lack of CYP1A1 induction. However, co-exposure with TCDD resulted in a significant decrease in TCDD-induced CYP1A1 (EROD) activity in these AhR-EGFP cell lines (Figure 3). These results are in agreement as those observed earlier with the rodent H4IIE rat hepatoma cell line, human hepatoma HepG2 and breast carcinoma MCF-7 cell lines [22]. For the lower

brominated PBDEs, like BDE-47, this decrease in EROD activity concurred with a decrease in AhR-EGFP reporter gene expression (Figure 3A, Figure 4A). In these instances, the antagonism in AhR-EGFP expression indicates that the antagonistic effect on TCDD-induced CYP1A1 by PBDEs is related to AhR binding and activation rather than simple competitive inhibition of EROD activity. This would also suggest that PBDEs could bind to the AhR as antagonists and compete with more potent agonists for binding the AhR in analogy with what was found for PCBs [35,36].However, some of the higher brominated PBDEs like BDE-183 also reduced TCDD induced CYP1A1 (EROD) activity (Figure 4C), while not influencing the AhR-EGFP gene expression (Figure 3C). This might suggest that some of these PBDEs could reduce EROD activity by virtue of their ability to act as competitive CYP1A1 substrates as has been observed for other chemicals such as the PCBs [35,36].

In our experiments the lateral substituted BDE-77 showed the strongest inhibitory effect on TCDD-induced CYP1A1 activity and AhR-EGFP expression. This clearly suggests that the substitution pattern of PBDE congeners that presumably bind the AhR is of importance for the observed antagonistic effects (the planarity of the congeners could facilitate binding to the AhR ligand binding domain). In analogy with the observed effects of the bromine substitution pattern, previously Suh et al (2003) reported that the chlorination substitution pattern of certain di-*ortho* substituted PCBs congeners determine their ability to elicit antagonistic effects on TCDD induced AhR-mediated effects [37]. Antagonistic effects by the higher chlorinated PBDEs were also observed at the XRE level. Binding to the XREs on the DNA was indirectly assessed by the use of synthetic reporter genes, which contain XRE binding sites in a heterologous promotor context, that reports the increased basal activity [38].

Binding to the XRE on DNA as indirectly measured in our experiments, suggests that some of these PBDEs are capable of initiating the AhR transformation as well as the nuclear localization. All PBDEs tested in our study elicited an inhibitory effect on TCDD-induced XRE-driven luciferase activity. However, based on our data it seems that the affinity of the higher brominated congeners is lower than that of the lower brominated congeners with regard to AhR-mediated effects.

A further analysis of the effects of BDE-99, the congener giving the most consistent and pronounced inhibitory effects in our present study, was performed with the Gal4-AhR construct. Singular exposure to BDE-99 did not lead to significant effects on the AhR, but the ChIP assay confirmed the ability of BDE-99 (10 µM) to allow the AhR to bind to the promotor region on the DNA (Figure 6).

It has been suggested that certain di-*ortho* substituted PCBs bind the AhR but show no CYP1A1 induction, depending on the substitution pattern [37,39,40]. However, this ligand binding to the AhR is probably unproductively as suggested previously for PCB-153 [39,40]. We suggest that, concerning the antagonistic effects of PBDEs with TCDD, there is a similar mechanism of action since ligand binding to the AhR alone is not sufficient for achieving later steps in the signaling pathway such as CYP1A1 induction, as was suggested earlier by Chen et al [39].

In summary, our results confirm previous findings that suggest PBDEs can bind, but not activate the AhR-XRE complex and subsequent transcription processes like that of CYP1A1 [22,23]. This effect appears to be most pronounced for the lower brominated congeners, which antagonize TCDD-dependent transcriptional activation by the AhR. The mechanism for this is unknown, but as BDE-99 can cause nuclear translocation of the AhR with transcriptional activation not being initiated, this compound could cause an unfavorable conformational change of the receptor that does not allow binding to co-factors and/or components of the initiation complex. Observed interactions for some of the higher brominated PBDEs on CYP1A1 activity may be AhR independent and likely occur by competitive inhibition of CYP1A1-dependent EROD activity, but this remains to be confirmed.
Since the concentrations of both TCDD and individual PBDEs in our *in vitro* experiments far exceed the current levels in human blood or plasma in Europe, it is impossible to draw conclusions from these antagonistic interactions between both groups of compounds for human health. However, the observed effects might still have implications for the risk assessment as humans are exposed to a complex mixture with a large number of dioxin-like compounds, PBDEs and PCBs. As many quantitatively important PBDEs and PCBs can apparently act as AhR antagonists and most likely could act in concert when present in mixtures, this could actually influence the overall effect of dioxin-like compounds in a down regulating way. Based on our *in vitro* results the question remains to which extent these PBDEs could actually inhibit or antagonize AhR mediated toxicological and biological effects *in vivo*, which should clearly be assessed further.

Acknowledgements
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CHAPTER FIVE

Effects of Polybrominated Diphenyl Ethers (PBDEs) on CYP3A4 induction in human hepatocytes (HepG2)

A.K. Peters, K. Gradin, Å. Bergman, M. Van den Berg, L. Poellinger

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Abstract

Polybrominated diphenyl ethers (PBDEs) are commonly used as brominated flame retardants and have been detected worldwide in biotic and abiotic samples. This study focused on the ability of individual environmentally relevant PBDE congeners (BDE-47, -77, -99, -100, -153, -154, -183) to induce Cytochrome P450-3A4 (CYP3A4) through activation of the human pregnane X receptor (hPXR). CYP3A4 is the main cytochrome P450 enzyme in the human liver and is responsible for the metabolism of many exogenous as well as endogenous compounds.

We studied the possible interaction between these PBDEs and hPXR, and the subsequent induction of CYP3A4 in human hepatoma (HepG2) cells transiently transfected with the hPXR or the cytochrome P450 CYP3A4 distal and proximal promoters plus the corresponding Gal4 luciferase reporter gene. At the highest concentrations tested (10 µM), the lower brominated BDE-47, -77, -99 and BDE-153 induced CYP3A4 luciferase activity significantly compared to the vehicle control DMSO (0.1%), resulting in a maximal induction of 2.5 ±0.2 fold for BDE-99. The positive control Rifampicin (0.01-10 µM) significantly induced CYP3A4, with a maximum of 2 ±0.3 fold at a concentration of 10 µM. However, TCDD (0.05-1 nM) was the most potent inducer in this system resulting in a CYP3A4 induction up to 7 ±0.2 times the vehicle control. At receptor level, the positive control Rifampicin (5 µM) resulted in a maximal induction of hPXR luciferase activity (9 ±2.7), followed by TCDD (1 nM, 5 ±0.5). Again, it was observed that the BDEs-47, -77, -99 (10 µM) could cause a small though significant ($p<0.05$) induction, with a maximum of 3.2 ±0.5 for BDE-77. These results were confirmed by quantitative RT-PCR. The *in vitro* concentrations of PBDEs used in this study however, exceeded the levels that are currently found in biota, including humans. The interaction between these PBDEs and *CYP3A4* is a novel finding with respect to mechanism of action of these compounds.

Introduction

Due to the increased use of synthetic consumer products in the household setting, the applications of fire safety standards have improved and flame retardants have become an increasing necessity. Polybrominated diphenyl ethers (PBDEs) are an important class of brominated flame retardants (BFRs) that are used as additives in various consumer products in plastic materials, paints, and textile fabrics since the 1970s. The possible substitution pattern of the bromines is like that of the chlorination pattern of PCBs with 209 possible congeners, ranging from mono- to deca-BDE. Similar as used for PCBs, the numbering of the PBDE congeners is adapted from the International Union of Pure and Applied Chemistry (IUPAC) system [1].

Worldwide there are three commercial mixtures of PBDEs in production, differing in their degree of bromination; Penta-, Octa-, and Deca-BDE.

Although the use of PBDEs has a clear benefit in the way that they reduce the chances of ignition and burning of materials, these compounds also have the ability to leach out of the polymers in which they are present. As a result of this leaching process, large scale production and use, these PBDEs have become ubiquitous environmental contaminants with increased levels measured in fish, wildlife [2-5], human blood, adipose tissue [6,7], and milk samples [8].

Structural similarities of PBDEs with other polyhalogenated aromatic hydrocarbons that bind to the aryl hydrocarbon receptor (AhR) such as certain PCBs, raised concern about the possible dioxin-like effects of these PBDEs. In previous studies, the ability of PBDEs to interact with the aryl hydrocarbon (Ah) receptor was studied. These studies provided some initial information that PBDEs could initiate AhR-mediated processes. However, the highly purified PBDE congeners were not able to initiate transcription of the *CYP1A1* gene [9,10]. Although PBDEs by itself did not seem to elicit significant dioxin-like transcriptional responses it was found that combinations with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) could result in significant antagonistic effects towards TCDD-induced effects. Similar interactive effects have been observed with specific non-dioxin-like PCBs, like PCB-153 [11,12]. As all environmental relevant PBDEs have multiple *ortho* bromine substitution patterns it was hypothesized that these PBDEs can have a possible mechanism of action that resembles that of the non-dioxin-like, multiple *ortho* substituted PCBs. Such a mechanism could also resemble that of p
phenobarbital (PB) with respect to cytochrome P450 induction pattern, i.e. CYP3A4 induction in humans [13-15].

The liver is the major site for metabolic activity of endogenous and exogenous compounds. CYP3A4 monooxygenase plays a major role in drug biotransformation in humans and is present in high concentrations in both the liver and intestine [16]. The induction of CYP3A4 transcription is the basis for a number of drug-drug interactions [17]. In humans, more than 50% of all used drugs, as well as other xenobiotics and endogenous substances, are metabolized by CYP3A4 into their (active) metabolites. From a risk assessment point of view it is therefore important to identify xenobiotics that can upregulate CYP3A4 gene expression and protein synthesis.

Previously, Sanders and co-workers (2005) reported the induction of CYP3A4 in rats that were orally administered PBDEs. Results from this study indicated that BDE-47, -99, and -153 (1-10 µM) were able to up-regulate CYP3A in rats at doses similar to that of the non-dioxin-like PCB-153.

It is assumed that PB-like PCBs induce CYP3A4 in humans by activation of the human pregnane X receptor (hPXR) [18-21]. The hPXR is activated by a wide variety of lipophilic compounds. After activation, hPXR binds to specific DNA sequences in the *CYP3A4* gene and functions as a transcription factor for gene regulation [22]. PXR regulates the metabolism and elimination of bile salts, steroids and xenobiotics from the body. The sequence of the PXR ligand binding domain differs between species [18,23]; therefore it is of importance to test compounds in human derived cell lines, since there could be large inter-species variability in this mechanism of action.

In the current study we have assessed the ability of individual environmentally relevant PBDE congeners (BDE-47, -99, -100, -153, -154, -183) [5] and the non-*ortho* substituted BDE-77 to interact with the *CYP3A4* gene in human hepatoma cells (HepG2).

Materials and Methods

Chemicals

The chemicals used were obtained from the following companies: 2,3,7,8-TCDD (>99% pure) was purchased from Cambridge Isotope Laboratories (Woburn, MA, USA); environmentally relevant PBDE congeners (BDE-47, -99, -100, -153, -154, -183; >98% pure) were synthesized and each congener was subjected to a specific purification on activated charcoal and Celite to remove possible impurities [24]. Besides using the earlier mentioned environmentally relevant PBDEs, BDE-77 was included in our experiments because of its resemblance to the planar PCB-77 due

to its lack of *ortho*-bromine and two adjacent bromine atoms on both aromatic rings. The HepG2 cell line was obtained from American Type Culture Collection (ATCC, Manassa, VA, USA). Cell culture medium RPMI1640 with glutamine and phenol red, phosphate buffered saline (PBS) and fetal calf serum (FCS) were obtained from Gibco BRL (Breda, The Netherlands). Fugene 6 was purchased from Roche (Mannheim, Germany), Luciferase cell culture Lysis buffer (5x) reagent was purchased from Promega (Madison, WI, USA), Luciferase buffer and ATP substrate were purchased from BioThema (Handen, Sweden). Quantitative RT-PCR reagents were obtained form PE Applied Biosystems (Nieuwerkerk a/d IJssel, The Netherlands). OptiMEM was from Invitrogen (Carlsbad, CA, USA); all other chemicals were obtained from Sigma Chemical Company (St. Louis, MO, USA).

Cell culture

The human hepatoma cell line HepG2 was cultured in RPMI medium with phenol red and glutamine, supplemented with 10% heat inactivated FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin.

Transient transfection and luciferase assays

The Gal4-hPXR and Gal4-hCYP3A4 constructs and the corresponding Gal4-Luc reporter gene were constructed as described by Bertilsson and co-workers [18,25]. Since the constructs are coupled to Gal4 promotor regions, the endogenous AhR expression will not interfere with the observed luciferase activity. Transient transfections into HepG2 cells were carried out in 12-well plates, using 150 ng of the expression plasmid and 200 ng of the luciferase reporter per well in OptiMEM. Fugene-6 transfection reagent was used according to the manufacturer's instructions (1.5 µl/well). Approximately 24h after transfection, the culture medium was changed with fresh media containing solvent control DMSO (0.1%), Rifampicin (0.1-10 µM), TCDD (0.001-1 nM), or PBDEs (1-10 µM). After 24h the transiently transfected cells were rinsed with PBS and 100 µl lysis buffer was added. The cell lysates were collected by scraping and transferred to a microcentrifuge tube. After brief vortexing, cell lysates were spun (2-5 min, 4°C, maximum rpm) and 30 µl of the supernatant was mixed with 100 µl Luciferin substrate and 100 µl ATP substrate. Luciferase activity was determined using a luminometer (Biotherm). The values were normalized to the protein concentrations measured according to the method of Bradford, with BSA as protein standard [26].

Quantitation of hCYP3A4 and hPXR mRNA Levels

Human CYP3A4 and hPXR mRNA levels were examined by real-time reverse transcriptase-polymerase chain reaction (RT-PCR). HepG2 cells were exposed for 24 hours to the designated compounds; DMSO (0.1%), Rifampicin (0.01-10 µM), TCDD (0.001-1 nM) and different PBDEs (1-10 µM).

RNA was isolated using the RNA Insta-Pure System according to the manufacturer's instructions. The amount of RNA was quantified spectrophotometrically (260/280 nm) and checked for DNA impurities with 3% agarose gel electrophoresis and ethidium bromide staining. Quantitative analysis of hCYP3A4 and hPXR mRNA levels was carried out using real-time PCR technology with beta-actin as the endogenous control. Primers and probes for CYP3A4 (forward primer 5'-TTG TCC TAC CAT AAG GGC TTT TGT; reverse primer 5'- AAA GGC CTC CGG TTT GTG A; probe VIC- AGT GTG GGG CTT TTA TGA TGG TCA ACA GC -TAMRA) and hPXR (forward primer 5'- CCC AGC CTG CTC ATA GGT TC; reverse primer 5'- GGG TGT GCT GAG CAT TGA TG; probe VIC- TGT TCC TGA AGA TCA TGG CTA TGC TCA CC –TAMRA) are described elsewhere [22]. Primers and probe for beta-actin (forward primer 5'-TCC TCC TGA GCG CAA GTA CTC; reverse primer 5'-CTG CTT GCT GAT CCA CAT CTG) were designed using Primer Express software (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Total RNA (10 ng) from the cells was amplified using a Taqman thermal cycler (7000 Sequence Detection System, ABI PRISM® Applied Biosystems). One-step RT-PCR Mastermix was used under the following conditions: 30 min at 48°C, 10 min at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 min at 60°C. Fluorescence data were processed and analyzed with ABI PRISM® Sequence Detection software (Applied Biosystems). The results of the PCR assay were expressed as fold induction of $\Delta\Delta C_T$ values compared to the vehicle control DMSO.

Statistical analysis

In each experiment exposure to the test compound was carried out in triplicate. Statistical differences among treatments were determined by a two-tailed Student t-test, with a significance level at >95% ($p<0.05$). Data in the figures are presented as one representative experiment (n=3, ±SD).

Results

After transient transfections with the Gal4-hCYP3A4 construct in human HepG2 cells, the positive control Rifampicin (0.1-10 µM) induced the luciferase activity significantly.

This resulted in a maximal induction of 2.0 ±0.3 fold compared to the vehicle control at a concentration of 10 µM (Figure 1). The same was observed after transient transfection with the Gal4-hPXR construct, resulting in a maximum of 9.2 ±2.7 (Figure 2). The lower brominated PBDEs, BDE-47, -77, -99, and BDE-153 were able to elicit significant ($p<0.05$) induction of hCYP3A4 at the highest concentration tested (10 µM) of 2 ±0.4, 1.6 ±0.1, 2.5 ±0.2, and 1.8 ±0.4 fold respectively (Figure 1). This concurred with a significant induction of hPXR of 1.9 ±0.1, 3.2 ±0.5, 2.0 ±0.03, and 1.8 ±0.02 fold respectively (Figure 2). Surprisingly, TCDD was a potent inducer of hCYP3A4 luciferase activity in the modified HepG2 cells, resulting in a 7-fold ±0.2 induction. This concurred with an increase of hPXR luciferase activity (5 ±0.5 fold).

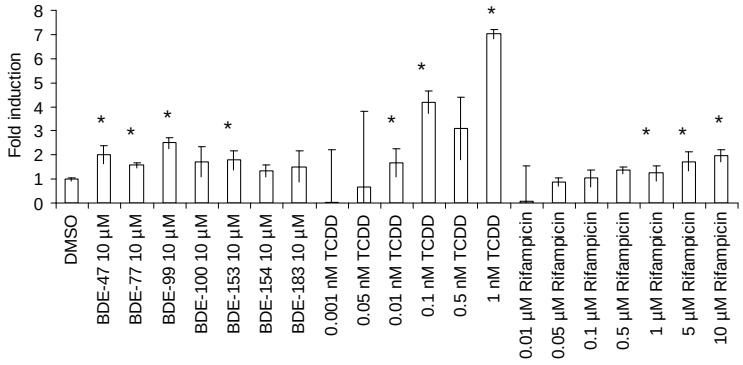

Figure 1; Effects of PBDEs on CYP3A4 expression in transiently transfected HepG2 cells. Data are expressed as one representative experiment (n=3, ±SD); * statistically significant compared to the solvent control DMSO (0.1%) ($p<0.05$)

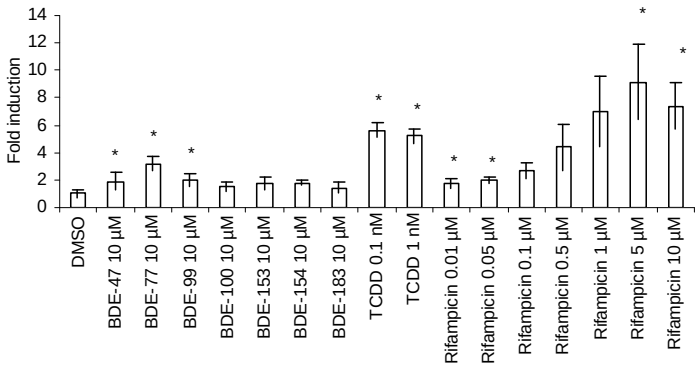

Figure 2; Effects of PBDEs on hPXR expression in transiently transfected HepG2 cells. Data are expressed as one representative experiment (n=3, ±SD); * statistically significant compared to the solvent control DMSO (0.1%) ($p<0.05$)

Quantification of hCYP3A4 mRNA resulted in a similar pattern as observed after the transient transfections (Figure 3). The positive control Rifampicin (10 µM) resulted in a $\Delta\Delta C_T$ value of 18 compared to the vehicle control. The maximum $\Delta\Delta C_T$ value was obtained for BDE-47 5 µM (45.7 fold induction). Other lower brominated PBDEs resulted in an induction of mRNA, though not always above background level. TCDD did not induce CYP3A4 mRNA.

Quantification of hPXR mRNA resulted in increased mRNA values; the positive control Rifampicin resulted in a 14.3 fold induction (Figure 4). BDE-47, -99, and -183 increased mRNA levels above background level, from which BDE-99 (10 µM) resulted in the highest mRNA levels (3.7 fold compared to the vehicle control). We could not confirm the previous findings of TCDD on hPXR expression in transiently transfected cells at hPXR mRNA level.

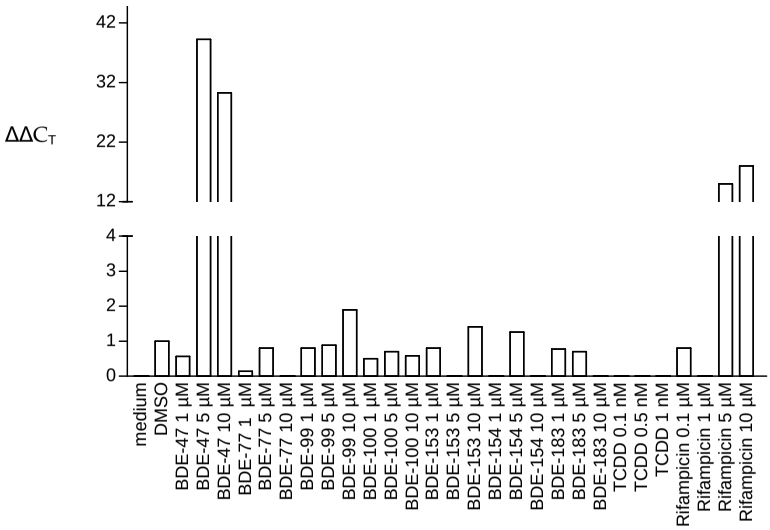

Figure 3; Effects of PBDEs on hCYP3A4 mRNA expression in HepG2 cells. Data are expressed as fold induction of ΔΔCT values compared to the vehicle control DMSO (0.1%).

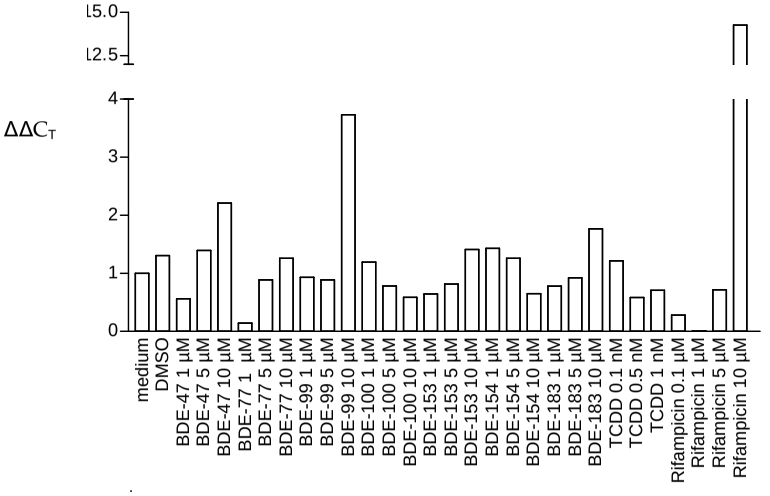

Figure 4; Effects of PBDEs on hPXR mRNA expression in HepG2 cells. Data are expressed as fold induction of ΔΔCT values compared to the vehicle control DMSO (0.1%).

Conclusion and Discussion

Several studies have addressed the question whether or not PBDEs interact with AhR and initiate receptor mediated transcriptional processes. In general, results of these studies have indicated that these brominated flame retardants are poor AhR agonists and some of the more pronounced observed AhR mediated responses have now been ascribed to the presence of impurities like brominated dibenzofurans [9,10,27-31]. As a consequence it is now generally accepted that PBDEs do not have to be included in the toxic equivalency (TEF) system that is globally used for complex mixtures of dioxin-like compounds [32,33]. However, it should be noted that some lower brominated PBDEs such as BDE-99 can interfere with the AhR-DNA pathway, without actually activating the associated transcriptional pathways like that for CYP1A1 [10]. Evidence has been recently provided that this non transcriptional interaction between PBDEs and the AhR could in fact be the basis for the antagonistic effects observed between e.g. TCDD and these PBDEs [10].
Thus, a comparison with PBDEs and dioxin-like compounds, including some planar PCBs like PCB-126, is clearly not supported by any evidence from a mechanistic point of view. From a structural point of view this lack of dioxin-like activity is not surprising as the environmental relevant PBDEs all have multiple *ortho* bromine atoms in common, which makes the attenuation of a planar structure unlikely.
This assumed non-planar configuration of these PBDEs has lead to the proposition that PBDEs could very well act as PB-like PCBs, like PCB-153, rather than being dioxin-like compounds. The mechanism of action and toxicology of non-dioxin-like PCBs has been less well defined, but includes aspects such as neurotoxicity and tumour promotion [34-37]. However, the cytochrome P450 PB-type of induction appears to be one of the more characteristic effects of these non-planar PCBs. In the rat, P450 induction by this group of PCBs is characterized by induction of the enzymes of the CYP2B and 3A families similar to that of PB, hence the expression PB-type of PCBs [37]. Indeed, Sanders and co-workers (2005) reported that the BDEs-47, -99, and -153 were able to upregulate both *CYP2B* and *CYP3A* gene expression in rats [27], confirming the possible structural similarity of PBDEs with these non-dioxin-like PCBs with respect to P450 induction. In the expression of both types of P450 families, large species differences have been observed. In addition, the expression of CYP2B enzymes is quantitatively important in rodent species, while in humans the expression of CYP3A4 and associated protein hPXR is quantitatively more important [38]. Species differences in CYP3A4 expression can, at

least in part, be explained by differences in the ligand-binding domain of the PXR, and not the DNA binding site [39]. This makes extrapolation of animal models to the human situation more complicated. Furthermore, the induction of *CYP3A4* gene expression by exogenous compounds is also known to be highly species dependent [17,40]. The results from our study and those mentioned above give further support to the idea that PBDEs can act as PB-type of inducers, such as some non-dioxin-like PCBs. The observation in our experiments that TCDD was also able to induce CYP3A4 in our genetically modified HepG2 cell system is rather remarkable and concurred with an increase in hPXR luciferase activity. However, we were not able to support these enzymatic findings at mRNA levels of hCYP3A4 and hPXR.

Our current study provides evidence that the observed induction of CYP3A in rats [27] can also occur in a human derived *in vitro* system, i.e. the human hepatoma HepG2 cells. Interference with the CYP3A4 enzyme system might e.g. have implications for drug-drug interactions, as this human P450 enzyme is the predominant drug metabolizing enzyme in the human liver [15,41]. As a consequence, binding of a xenobiotic compound to hPXR might be useful to predict drug interactions. It has been suggested that substances which bind to hPXR should be removed from drug development processes and should in generally be avoided [42]. However, when evaluating our results from a human risk assessment perspective, it should be realized that the observed *in vitro* effects occurred at medium concentrations (10 µM) that exceed the current human plasma levels of PBDEs.

Assuming that medium concentrations have some predictive value for levels observed in European human plasma it appears that induction of CYP3A4 by PBDEs is not expected in normal background exposure situations.

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CHAPTER SIX

Determination of *In Vitro* Relative Potency (REP) Values For mono-*ortho* Polychlorinated Biphenyls after Purification with Active Charcoal

A.K. Peters, P.E. Leonards, B. Zhao, Å. Bergman, M.S. Denison, M. Van den Berg

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Abstract

The Toxic Equivalency factor (TEF) system for dioxin-like compounds has included assignment of TEF values for mono-*ortho* polychlorinated biphenyls (MO-PCBs) in mammals. However, when testing these compounds for their ability to elicit dioxin-like responses, it has recently been suggested that small traces of aryl hydrocarbon receptor (AhR)-active impurities could result in artifactually higher relative potency (REP) values than reported so far. MO-PCBs 2,3,3',4,4'-pentachlorobiphenyl (PCB-105), 2,3',4,4',5-pentachlorobiphenyl (PCB-118), 2,3,3',4,4',5-hexachlorobiphenyl (PCB-156), and 2,3',4,4',5,5'-hexachlorobiphenyl (PCB-167) were purified on an active charcoal column commonly used in analytical chemistry to remove any planar AhR agonists like chlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) that could be present as impurities. The ability of these purified MO-PCBs to activate or inhibit AhR-dependent gene expression was studied in two stably transfected rodent hepatoma cell lines (H1G1.1c3 mouse and H4G1.1c2 rat hepatoma cells) containing an AhR-responsive enhanced green fluorescent protein (AhR-EGFP) reporter gene. In addition, effects on 7-ethoxyresorufin-O-deethylation (EROD) activity were used as a marker for CYP1A1 activity. MO-PCBs -105, -118 and -156 induced AhR-EGFP expression in both rodent cell lines. PCB-156 (10 µM) was the most efficacious MO-PCB, inducing reported gene expression to a maximum of ~27% of that of a maximal inducing concentration of TCDD in the mouse cell line and to 62.5±3.4% in the rat cell line. This was accompanied by an increase in EROD activity in mouse and rat cell lines to a maximum of 20.5±1.5% and 68±3.2% of TCDD, respectively. No AhR-mediated induction was observed for PCB-167. Both the EGFP expression as well as the CYP1A1 induction occurred at very high concentrations (≥ 0.1 µM) compared to TCDD (≥ 0.001 nM), resulting in REP values that are ~10 to ~120 times lower than current WHO values.

In the H1G1.1c3 mouse cell line significant reduction of TCDD-induced AhR-EGFP expression were also observed for PCB-105, -118 and -156 up to 50.9±2.9%, 58.3±2.2% and 70.8±1.3% of TCDD at PCB concentrations of 10 µM. These results were confirmed on CYP1A1 level and also resulted in reduction of TCDD-induced EROD activity of 39.3±2.8%, 67±5% and 48.3±4% compared to TCDD. PCB-167 was almost inactive on TCDD-induced responses, and did not result in any significant reduction. In the rat cell line, only PCB-156 resulted in a significant decrease in TCDD-induced AhR-EGFP expression of 35%, suggesting that species differences play an important role in this reduction. Our results suggest that purification of

MO-PCBs is an essential step in determining accurate REP values, and could very likely leads to lower TEF values than those presently assigned by the WHO.

Introduction

Polychlorinated dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs) and biphenyls (PCBs) are important classes of persistent organic compounds found in the global environment and many individual congeners are highly toxic to living organisms [1]. Because of their insulating and flame retardancy properties, PCBs were extensively used in industrial applications, and have been commercially produced since the 1920's. Releases of PCBs into the environment have resulted in a widespread global distribution of PCBs into all environmental and biological matrices [2]. These compounds are lipophilic and accumulate in lipid rich body tissues and fluids from man and wildlife species and can biomagnify up the food chain. PCBs were banned in open applications in many countries in the Western world since the late 1970s due to their persistent nature and potent toxic effects [3]. PCB exposure, especially in occupational settings and wildlife, has been associated with e.g. chloracne, carcinogenesis, teratogenesis, impaired immune responses, endocrine disruption, as well as the induction of gene expression [1,4]. The induction of cytochrome P450 enzymes (e.g. CYP1A1) has been shown to be produced by dioxin-like compounds such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) [1,5,6].

Environmental contamination by PCDDs and PCDFs has occurred trough emission from waste incinerators and general combustion reactions, their presence as impurities in industrial products such as herbicides and generation during the bleaching of wood pulp for paper production [1,4,7]. Human exposure to PCBs and dioxin-like compounds occurs primarily through occupational and dietary exposure, although concentrations of PCBs and dioxins have declined over the last decades [5,8,9].

There are 209 possible PCBs congeners and their dioxin-like biological and toxic effects and relative potency depend on their chlorination pattern which dictates their ability to bind to and activate the aryl hydrocarbon (Ah) receptor and AhR-dependent toxicity [1,5,6,10,11]. In this regard, PCBs have been structurally divided into three groups; the non-*ortho* PCBs with a planar conformation, ability to activate the AhR and produce dioxin-like effects; the mono-*ortho* (MO-) substituted PCBs (e.g. PCB-105, -118, -123, -156, -157, -167, and -189) with some planar conformation, weak ability to activate the AhR and produce some dioxin-like

effects; and multiple-*ortho* substituted PCBs with a non-planar configuration, no or very low affinity for the AhR and inability to produce dioxin-like effects. The biological and toxic effects of PCBs have been studied extensively and have primarily focused on the non-*ortho* substituted congeners i.e. PCBs -77, -126 and -169 as these are potent agonists for the AhR that produce dioxin-like toxic effects and consequently have been assigned toxic equivalency factors (TEF) values by the World Health Organization (WHO) [5,7,10]. While the MO-PCBs have always been thought to have low affinity for the AhR, with TEF values varying from $1x10^{-5}$ to $1x10^{-4}$ [1,5-7], due to their relatively high concentrations in food and human milk these PCBs contribute significantly to the overall total Toxic Equivalency (TEQs) of a sample [4,5]. The MO-PCBs, together with the non-*ortho* substituted dioxin-like PCBs typically represent a higher percentage of the total TEQs than that from the PCDDs and PCDFs [5].

The toxic and biological effects of PCDDs, PCDFs and those PCBs with dioxin-like activity are mediated by the AhR, a ligand-dependent transcription factor. While the AhR can be found in the cytoplasm of almost all vertebrate cells, significant species and tissue differences exist in its concentration and distribution [12-14]. In addition to the planar halogenated polyaromatic compounds, recent studies have revealed the ability of very structurally diverse chemicals to bind and/or activate the AhR and AhR-dependent gene expressions [12,13,15]. Upon ligand binding, the ligand:AhR complex translocates into the nucleus, wherein it binds to the AhR nuclear translocator protein (ARNT) [16]. Binding of the resulting ligand:AhR:ARNT complex to its specific DNA recognition site, the dioxin response element (DRE), stimulates transcription of a variety of genes, including that of Cytochrome P450 1A1 (*CYP1A1)* [17-21].
The induction of CYP1A1 has been used both as a model system to study the molecular mechanism of AhR activation and as a bioanalytical method to determine the relative potency of AhR agonists [22]. Given the role of the AhR in both gene induction and toxicity of these chemicals, it is not surprising that there is a high degree of correlation between the relative potency (REP) of PCDDs, PCDFs and PCBs to induce AhR-dependent gene expression (e.g., CYP1A1) and their relative toxic potency (i.e., their TEF value).
The ability of MO-PCBs to induce CYP1A1 has been previously examined in various human cell lines. While MO-PCBs failed to induce EROD activity in the human hepatoma (HepG2) cell line, or in a human prostate carcinoma (LNCaP) cell line, they were weak inducers of CYP1A1 in the rat hepatoma (H4IIE) cell line

[23,24]. In addition, although PCB-156 could induce CYP1A1 in fish (PLHC-1) hepatoma cells, two other MO-PCBs, -105 and -118, induced little or no CYP1A1 activity [25]. In primary cultures of rat and cynomolgus monkey hepatocytes, MO-PCBs were found to induce CYP1A1-dependent EROD activity [23,26-28].

In contrast, no CYP1A1 induction was observed with individual MO-PCBs (except PCB-156) in *in vivo* experiments with rats and mice [29,30]. Thus, when reviewing the relative potencies of MO-PCBs in various *in vitro* and *in vivo* systems there are significant, yet unexplained differences, which are in strong contrast with consistent induction results obtained with PCB-126 [31,32].

While species, tissue and cell-type differences could contribute to the variability in the results of the above studies, recent questions regarding the presence of impurities in MO-PCB preparations that have relatively potent dioxin-like activities have complicated clear interpretation of prior studies [7,33]. The presence of low levels of potent AhR-active compounds (such as PCDFs or coplanar PCBs) as impurities in a test compound that is presumed to be pure, could actually be responsible for some or all of the AhR-mediated responses that would otherwise be contributed to the test compound [34,35].

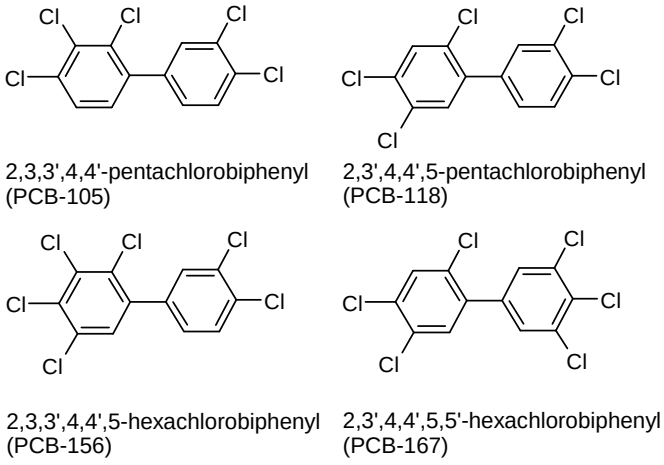


Figure 1; Structure of the compounds used in this study

Here we have used several MO-PCBs (PCB-105, -118, -156, and -167; Figure 1) that have been extensively purified to eliminate the presence of dioxin-like PCDDs, PCDFs and PCB contaminants This was done in order to clearly determine whether these MO-PCBs can bind to and activate AhR-dependent gene expression

and/or whether their previously documented ability to activate the AhR was due to the presence of other AhR-active agonist contaminants present in the MO-PCB preparations.

Materials and methods

Chemicals

The mono-*ortho*-PCBs -105, -118, -156, and -167 were purchased from Cambridge Isotope Laboratories (Woburn, MA, USA); each congener was subjected to purification on activated charcoal and Celite to remove possible contamination with undesired dioxin-like contaminants [36]. The compounds were subsequently analyzed by HRGC-MS to determine whether the contaminants were still present in the mixture.

2,3,7,8-TCDD (>99% pure) was purchased from Cambridge Isotope Laboratories (Woburn, MA, USA); cell culture media Dulbecco's Modified Eagle Medium (DMEM), phosphate buffered saline (PBS), and fetal calf serum (FCS) were obtained from Gibco BRL (Breda, The Netherlands). All other chemicals were obtained from Sigma Chemical Company (St. Louis, MO, USA).

Cell culture

The mouse and rat hepatoma cell lines (H1G1.1c3 and H4G1.1c2) were created by stable transfection of mouse hepatoma (Hepa1c1c7) and rat hepatoma (H4IIE) cells with the AhR-responsive Enhanced Green Fluorescent Protein (AhR-EGFP) reporter plasmid pGreen1 as reported earlier [37]. Both cell lines were cultured in DMEM supplemented with 10% heat inactivated FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin in an incubator (5% CO_2, 37ºC).

Enhanced green fluorescent protein (EGFP) assay

The H1G1.1c3 and H4G1.1c2 cell lines were stably transfected with an enhanced green fluorescent protein (EGFP) reporter containing approximately 500 bp of the CYP1A1 promoter including 4 XRE sequences [37]. Both cell lines were seeded in normal culture medium in 96 well plates ($1x10^4$ cells/well) and exposed after 24h to the solvent control DMSO (0.1%), positive controls TCDD (0.001-1 nM) and PCB-126 (0.01-10 nM), the indicated MO-PCBs (0.01-10 µM), or co-exposed to concentrations of both TCDD and MO-PCBs. After 72 hours the cells were washed twice with phosphate buffered saline (PBS, 37°C), PBS was added to each well and fluorescence of the intact cells was measured using an excitation wavelength of

485 nm and emission wavelength of 510 nm in a Fluostar (BMG). Induced AhR-EGFP activity was determined by subtracting the background fluorescence in the DMSO sample from the fluorescence treated samples [38].

7-Ethoxyresorufin-O-deethylase (EROD) assay

Ethoxyresorufin-O-deethylation (EROD) activity was used as a marker for CYP1A1 catalytic activity using a modification of the method described by Burke and Mayer (1974) as we have recently reported [39]. Directly following the AhR-EGFP assay, the PBS in the well plates was replaced with serum-free medium supplemented with 5 mM $MgCl_2$, 5 mM 7-ethoxyresorufin (7-ER), and 10 mM dicumarol. Metabolic conversion of 7-ER into the fluorescent resorufin product was followed over a 10 min period at 37ºC using an excitation wavelength of 530 nm and emission wavelength of 590 nm.

Cell viability

After cells were incubated with the designated compounds for 72h, they were washed and medium was replaced with a 1 mg/ml MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) solution. The formation of MTT to formazan in the presence of the mitochondrial enzymes succinate dehydrogenase was allowed for 1h at 37°C [40]. After the incubation period, cells were washed and the formazan was extracted by adding 0.1 ml of isopropanol and measured spectrophotometrically (595 nm). The results were expressed as % of cell viability compared to the vehicle control DMSO (100% viable cells).

Statistical analysis

All experiments were carried out three times, in triplicate. Statistical differences among treatments were determined by a two-tailed Student t-Test, with a level of statistical significance of 95% ($p<0.05$).

Results

Singular exposure with either TCDD or PCB-126 or the MO-PCBs did not result in any cytotoxicity in either cell lines as measured with the MTT assay. The highest concentration tested for the PCBs was 10 µM since higher concentrations resulted in a visible suspension, likely resulting from the limited solubility of the chemicals. Co-exposure of the cells to TCDD and PCB-167 resulted in a significant decrease in

cell viability for TCDD (≥ 0.1 µM) and PCB-167 at 10 µM and thus, these concentrations were therefore excluded from the experiments.

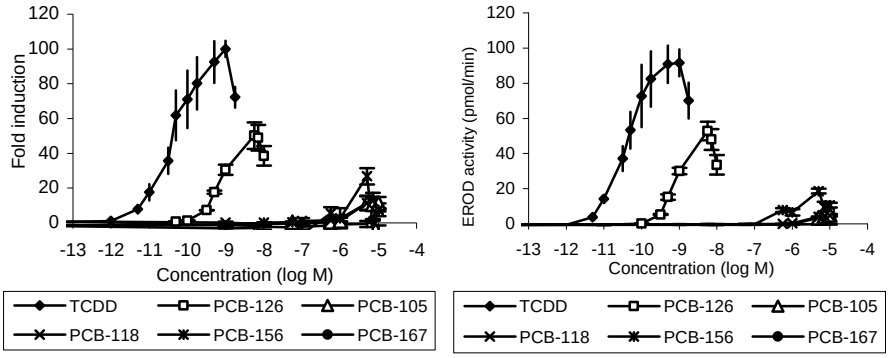


Figure 2; Induction of AhR-dependent gene expression by TCDD, PCB-126 and MO-PCBs in recombinant mouse hepatoma cells. H1G1.1c3 cells were incubated with DMSO, TCDD (0.001-1 nM), PCB-126 (0.001-10 nM) or mono-*ortho* PCBs (0.001-10 µM) for 72 hours and AhR-EGFP expression (on the left) or EROD activity (on the right) measured as described in the Materials and Methods. The data are expressed as mean ± SEM (n=3).

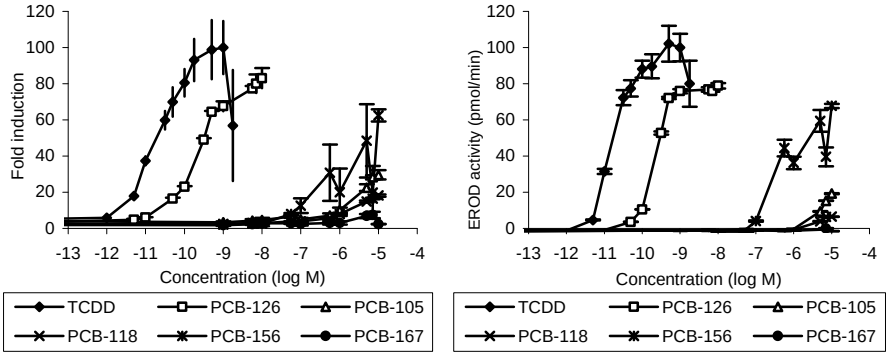


Figure 3; Induction of AhR-dependent gene expression by TCDD, PCB-126 and MO-PCBs in recombinant rat hepatoma cells. H4G1.1c2 cells were incubated with DMSO, TCDD (0.001-1 nM), PCB-126 (0.001-10 nM) or mono-*ortho* PCBs (0.001-10 µM) for 72 hours and AhR-EGFP expression (on the left) or EROD activity (on the right) measured as described in the Materials and Methods. The data are expressed as mean ± SEM (n=3).

Agonistic effects of mono-*ortho* PCBs on AhR-EGFP expression

Both TCDD and PCB-126 induced strong AhR-EGFP expression in both mouse H1G1.1c3 and rat H4G1.1c2 cells to a maximum for PCB-126 of 50±7.7% and 83±5.6% respectively, compared to TCDD (100%) (Figure 2A, 3A, Table 2). EC_{25} and EC_{50} values for TCDD and PCB-126 were comparable between the two cell lines (Table 2) with REP values for PCB-126 of 0.05 (EC_{50}) and 0.07 (EC_{25}) in the mouse cell line and 0.04 (EC_{50}) and 0.07 (EC_{25}) in the rat cell line (Table 2).

The MO-PCBs PCB-105, -118, -and -156 produced a significant induction of AhR-EGFP expression in the mouse cell line to a maximum of 11.4±3.5%, 7.5±1.0%, and 26.8±4.7%, respectively. A higher induction was observed in the rat cell line of 29.6±2.6%, 18.2±0.6%, and 62.5±3.5% that of TCDD, respectively (Table 2). Since the MO-PCBs did not reach a maximal induction compared to TCDD, calculation of the EC_{50} or EC_{25} was merely theoretical and considered not a realistic comparison with TCDD. Therefore, $EC_{5\ TCDD}$ values were also calculated, as previously proposed by Behnisch and co-workers [41]. PCB-156 was the most efficacious inducer, resulting in REP values of 0.000004 ($EC_{5\ TCDD}$) in the mouse cells (tale 1), and 0.000025 ($EC_{5\ TCDD}$) in the rat cell line (Table 2). This is respectively 125 to 20 times lower than the current WHO value of 0.0005 [7]. PCB-105 resulted in 100 (mouse cell line, Table 1) to 50 (rat cell line, Table 2) times lower REP ($EC_{5\ TCDD}$) values than the current WHO value of 0.0001. PCB-118 also resulted in 50 to 100 times lower REP ($EC_{5\ TCDD}$) values than the WHO value of 0.0001, in the mouse and rat cell line, respectively (Table 1, 2).

Agonistic affects of mono-*ortho* PCBs on EROD activity

As observed in the AhR-EGFP assay, both TCDD and PCB-126 induced EROD activity in mouse and rat cell lines to a maximum for PCB-126 of 58±5.6% and 79±4.1% respectively, compared to TCDD (100%) (Figure 2A, 3A, Table 1, 2). EC_{25} and EC_{50} values for TCDD were $6.3x10^{-12}$ M and $1.9x10^{-11}$ M respectively in the mouse cells, and $8.9x10^{-12}$ M and $1.8x10^{-11}$ M respectively in the rat cells. For PCB-126, these values were $7.9x10^{-11}$ M and $2.2x10^{-10}$ (EC_{25} and EC_{50} values respectively) in the mouse cells, and $1.3x10^{-10}$ M and $2.1x10^{-10}$ M (EC_{25} and EC_{50} values respectively) in the rat cells (Table 2). This indicates a relative potency for PCB-126 of approximately 0.08 based on both EC_{50} and EC_{25} values.

Similar to results obtained in the AhR-EGFP assay, PCB-167 failed to induce EROD activity. PCB-156 was also the most efficacious inducer of EROD activity, reaching a maximum 20.5±1.5% of TCDD (100%) at a concentration of 5 µM in the mouse cells, and 68.0±3.2% of TCDD (100±21%) at a concentration of 10 µM (Table 1, 2) in

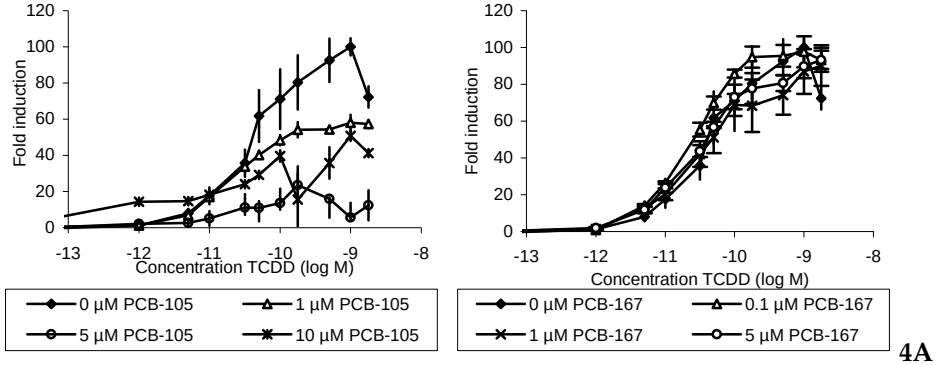
the rat cells. As the MO-PCBs did not reach a maximal EROD induction compared to TCDD, calculation of the EC_{50} or EC_{25} was merely theoretical and considered not a realistic comparison with TCDD. Therefore, $EC_{5\ TCDD}$ values were again calculated, as previously proposed by Behnisch and co-workers [41]. Corresponding REP values for PCB-156 were 0.000019 ($EC_{5\ TCDD}$) in the mouse cell line (Table 1) and 0.000045 ($EC_{5\ TCDD}$) in the rat cell line (Table 2), 26 to 11 times lower than the WHO value of 0.0005 respectively. REP values for PCB-105 were <0.000001 (mouse cell line, Table 1) to 0.000002 (rat cell line, Table 2) based on the $EC_{5\ TCDD}$ values, which is 100 to 50 times lower than the WHO values respectively. PCB-118 resulted in REP values of <0.000001 to 0.000001 based on $EC_{5\ TCDD}$ values in mouse and rat cells respectively, both 100 times lower than the WHO value of 0.0001 (Table 1, 2).

Antagonistic effects of mono-*ortho* PCBs on AhR-EGFP expression

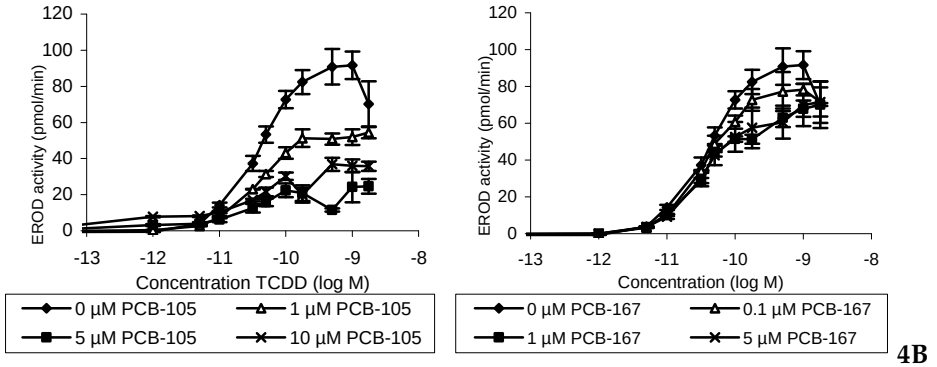
In contrast to their agonist effects on AhR-mediated gene expression, were more efficacious inducers in rat cells, antagonistic effects of MO-PCBs were more pronounced in the mouse cell line. Co-exposure of TCDD (0.001-1 nM) and MO-PCBs (0.1-10 µM) resulted in repression of TCDD-induced AhR-EGFP expression in the mouse cell line, PCB-167 (1-5 µM) and PCB-156 (1-10 µM) had minimal effects on TCDD-induced expression, while PCB-105 (1-10 µM) and PCB-118 (1-10 µM) reduced the TCDD-induced expression significantly in the mouse cell line (Figure 4A) with 10 µM inhibiting to a maximum of 50.9±2.9% and 58.3±2.2% respectively (Table 3). In the rat cell line, no significant dose-dependent antagonistic effects on AhR-EGFP expression were observed at the highest PCB concentrations (Figure 5A, Table 3). The EC_{25} and EC_{50} values were essentially unaffected by co-incubation with TCDD/PCB (Table 3); the main inhibitory effect was observed on the maximal level of inducible AhR-EGFP expression.

Antagonistic effects of mono-*ortho* PCBs on EROD activity

As observed after the AhR-EFGP assay, co-incubation of TCDD (0.001-1 nM) with MO-PCB-105, -118 and -156 resulted in concentration-dependent antagonistic effects on TCDD-induced EROD-activity in the mouse cell line (Figure 4B) with maximal inhibition of 26.7±9.3% for PCB-105 (5 µM), 46.5±10.4% for PCB-118 (5 µM) and 48.3±4% for PCB-156 (10 µM) compared to TCDD (100%) (Table 3). In contrast, no significant antagonistic effects on EROD activity were observed in the rat cell line.

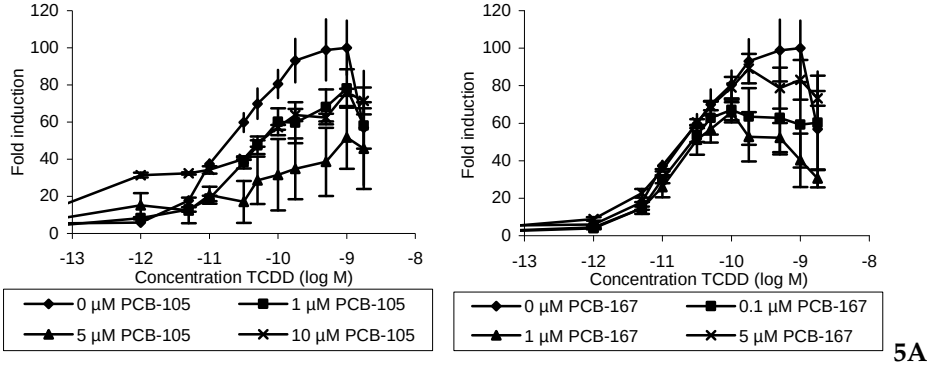


4A

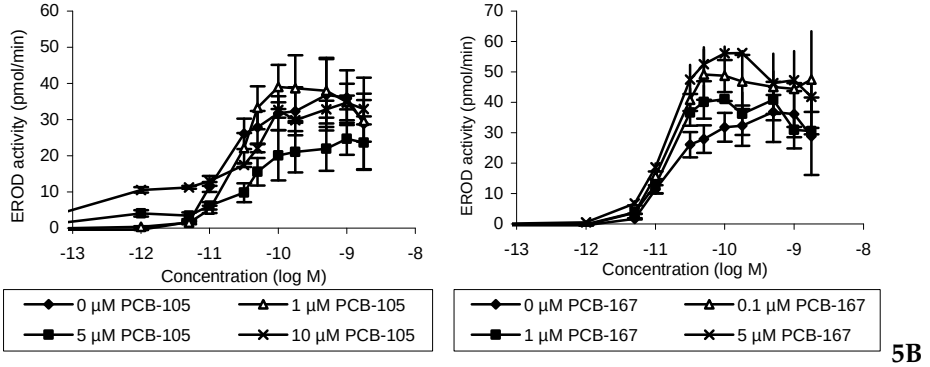


4B

Figure 4; Effects of MO-PCB-105 and -167 on the induction of AhR-dependent gene expression by TCDD in recombinant mouse hepatoma cells. H1G1.1c3 cells were incubated with DMSO, TCDD (0.001-1 nM), TCDD and PCB-105 (0-10 µM) or TCDD and PCB-167 (0-5 µM) for 72 hours and AhR-EGFP expression (A) or EROD activity (B) measured as described in the Materials and Methods. The data are expressed as mean ± SEM (n=3).



5A



5B

Figure 5; Effects of MO-PCB -105 and -167 on the induction of AhR-dependent gene expression by TCDD in recombinant rat hepatoma cells. H4G1.1c2 cells were incubated with DMSO, TCDD (0.001-1 nM), TCDD and PCB-105 (0-10 µM) or TCDD and PCB-167 (0-5 µM) for 72 hours and AhR-EGFP expression (A) or EROD activity (B) measured as described in the Materials and Methods. The data are expressed as mean ± SEM (n=3).

Discussion and Conclusion

In our current study the MO-PCBs -105, -118, -156 induced both low levels of AhR-EGFP expression as well as low EROD activity in both rat and mouse cell lines at concentrations approximately ≥ 0.1 µM (Figure 2, 3). This indicates and confirms that these MO-PCBs can cause AhR-mediated *in vitro* effects in both rodent species, even after an extensive clean-up procedure to remove more efficacious AhR agonist, though with very low potency and at high concentrations. The lack of AhR-mediated effects of PCB-167 could be caused by the chlorine substitution pattern. Other authors have found the chlorination pattern to be of importance with respect to the AhR mediated responses of PCBs [1,42,43].

Furthermore, high affinity ligand binding appears to be dependent upon key electronic and thermodynamic characteristics of the ligand [44-46].

The *in vitro* and *in vivo* experimental data that have been obtained concerning the toxicity of MO-PCBs, has resulted in their inclusion by the World Health Organization (WHO) in the dioxin-TEQ approach [4,7]. The TEF values that are published by the WHO for risk assessment in humans and animals are consensus values derived from numerous REPs derived from different *in vivo* and *in vitro* studies [47].

The WHO TEF values for MO-PCBs vary from $1x10^{-5}$ for PCB-167 to $1x10^{-4}$ (PCB-105 and PCB-118) [7]. When comparing the REP values obtained from our experiments (Table 1, 2) with the TEF values assigned by the WHO [7], we observed a 11 to 125 fold difference (based on $EC_{5\ TCDD}$ values as observed for AhR-EGFP expression of PCB-156 in the rat H4G1.1c2 cell line and for EROD activity after exposure to PCB-156 in the mouse H1G1.1c3 cell line. If these *in vitro* endpoints in both rodent species are assumed to correlate with dioxin-like toxicity of MO-PCBs, the suggested WHO TEFs [4,7] could be an overestimation of approximately one to two orders of magnitude.

In a recent study, Haws and co-workers (2005) reported a range of REP values obtained from various *in vitro* and *in vivo* experiments for PCBs [31]. The reported *in vitro* (REP_{2004} database) range for PCB-105 was $7.5x10^{-6}$ to 0.015 with the WHO TEF value in the 36th percentile. Our observed REP values are within the 10-25th percentile of these values. For PCB-118, the reported *in vitro* range was $6x10^{-6}$ to 0.0011, with the WHO TEF value in the 74th percentile and our REP values below the 50th percentile. For PCB-156, the reported *in vitro* range was $3x10^{-5}$ to 0.0014; the WHO TEF value was in the 43rd percentile and our observed REP values are also below the 50th percentile [32]. Clearly, the large variability between the reported REPs is not only due to the differences between *in vitro* and *in vivo* data and species differences, but also due to the differences in endpoints. The endpoints included in this extensive database can vary from biochemical changes to carcinogenicity studies [32].

Antagonistic effects of AhR-active PCBs have been reported previously from both *in vivo* as well as *in vitro* studies [1,42,48-52]. In our *in vitro* mixture experiments antagonistic effects of MO-PCBs (1-10 µM) were observed in the mouse H1G1.1c3 cells (Figure 4). Surprisingly, in the rat H4G1.1c2 cells, these MO-PCBs had only agonistic potencies (Table 1, Figure 3); no antagonistic effects were observed after exposure in combination with TCDD (Table 3).

Aarts and co-workers (1995) previously reported a similar finding with mouse cells being more sensitive than rat and human-derived hepatocyte cell lines in respect to AhR-mediated antagonistic effects of di-*ortho* PCBs.

With respect to human relevance of the observed AhR-mediated effects of MO-PCBs, it should be noted that medium concentrations in our *in vitro* experiments causing minimal effect were found around 0.1-1 µM. In human blood, concentrations of MO-PCBs are observed in the nM-range [53-55], Assuming *in vitro* medium concentrations can be used as surrogate for blood concentrations, a difference of two to three orders of magnitude is still present. This indicates AhR mediated effects of MO-PCBs might not easily be achieved in humans.

With respect to the observed antagonism between TCDD and some of these MO-PCBs it should be noted that these were found at ratios $1x10^4$ and $1x10^5$ that are also found in human samples like blood and milk [54-56]. Thus, the question remains to which extent the observed antagonism could have relevance for the human situation. Here we have to keep in mind that our *in vitro* experiments were performed at lower concentrations MO-PCBs, and in addition, we observed strong differences between mouse and rat cells.

There are several criteria for a compound that should be met for inclusion in the TEF concept: 1) the compound must bind the Ah receptor; 2) it should elicit dioxin-specific biochemical and toxic responses; and 3) be persistent and accumulate within the food chain [4,7]. Toxic equivalencies (TEQs) can be derived from complicated mixtures of dioxin-like compounds by multiplying the congener specific concentration with their individual TEF values. When used in this way for risk assessment, additivity is a prerequisite and this is supported by numerous *in vivo* and *in vitro* studies. Although, as in our study, antagonism or synergism have been reported for mixtures of dioxin-like compounds and PCBs, the magnitude of these non-additive effects is considered not to comprise this TEF concept significantly when e.g. compared to differences between species [7]. Results from our present study show that MO-PCBs should indeed be considered as weak AhR agonists and inclusion in the TEF concept is valid, but their present WHO TEF values might be too high. Nevertheless we question if MO-PCBs used in earlier studies have been sufficiently checked for impurities with potent AhR agonists like 2,3,7,8-substituted PCDDs, PCDFs, or planar PCBs such as PCB-126. It should be noted that earlier studies with PCBs or polybrominated diphenyl ethers have also indicated that these type of impurities could have significant influence on the outcome of a study when using weak AhR agonists [10,57].

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Effects of mono-*ortho* PCBs on AhR-EGFP expression in mouse H1G1.1c3 cells

Compound	$EC_{5\ TCDD}$ (nM)	EC_{25} (nM)	EC_{50} (nM)	Concentration † (nM)	AhR-EGFP			REP value ($EC_{5\ TCDD}$)	REP value (EC_{25})	REP value (EC_{50})
TCDD	$2.51*10^{-12}$	$1.58*10^{-11}$	$3.98*10^{-11}$	1	100	± 4.7	*	1	1	1
PCB-126	$2*10^{-10}$	$3.98*10^{-10}$	$7.50*10^{-10}$	5 (µM)	50	± 7.7	*	0.01	0.08	0.05
PCB-105	$1.78*10^{-6}$			10	11.4	± 3.5	*	0.000001		
PCB-118	$1.58*10^{-6}$			10	7.5	± 1.0	*	0.000002		
PCB-156	$5.60*10^{-7}$	$1.3*10^{-6}$		5	26.8	± 4.7		0.000004	0.000023	
PCB-167					nd					

Effects of mono-*ortho* PCBs on EROD activity in mouse H1G1.1c3 cells

Compound	$EC_{5\ TCDD}$ (nM)	EC_{25} (nM)	EC_{50} (nM)	Concentration † (nM)	EROD			REP value ($EC_{5\ TCDD}$)	REP value (EC_{25})	REP value (EC_{50})
TCDD	$5.60*10^{-12}$	$6.3*10^{-12}$	$1.9*10^{-11}$	1	100	± 8.4	*	1	1	1
PCB-126	$3.20*10^{-10}$	$7.9*10^{-11}$	$2.2*10^{-10}$	5 (µM)	58.2	± 5.8	*	0.02	0.04	0.1
PCB-105	$<1*10^{-5}$			10	5.6	± 0.63	*	<0.000001		
PCB-118	$<1*10^{-5}$			10	5.2	± 0.67	*	<0.000001		
PCB-156	$2.99*10^{-7}$			5	20.5	± 1.5	*	0.000019		
PCB-167					nd					

† *concentration resulting in maximal induction*

** statistically significant compared to the vehicle control (DMSO 0.1%) ($p<0.05$)*

Table 1; Effects of mono-*ortho* PCBs, TCDD, and PCB-126 on AhR-EGFP expression and EROD activity in stably transfected mouse hepatoma (H1G1.1c3) cells. The data are presented as one representative experiment (± SEM), conducted in triplicate.

Effects of mono-*ortho* PCBs on AhR-EGFP expression in rat H4G1.1c2 cells

Compound	$EC_{5\ TCDD}$ (nM)	EC_{25} (nM)	EC_{50} (nM)	Concentration ^{T} (nM)	AhR-EGFP		REP value ($EC_{5\ TCDD}$)	REP value (EC_{25})	REP value (EC_{50})
TCDD	$1.78*10^{-12}$	$1.50*10^{-11}$	$4.0*10^{-11}$	1	100	± 14.7 *	1	1	1
PCB-126	$2*10^{-10}$	$4.5*10^{-10}$	$8.9*10^{-10}$	5	83	± 5.6 *	0.01	0.07	0.04
				(µM)					
PCB-105	$1*10^{-6}$	$5.6*10^{-7}$		10	29.6	± 2.6 *	0.000002	0.000004	
PCB-118	$1.58*10^{-6}$			10	18.2	± 0.6 *	0.000001		
PCB-156	$7.08*10^{-8}$	$1*10^{-7}$	$2.8*10^{-7}$	5	62.5	± 3.4 *	0.000025	0.000032	0.000140
PCB-167					nd				

Effects of mono-*ortho* PCBs on EROD activity in rat H4G1.1c2 cells

Compound	$EC_{5\ TCDD}$ (nM)	EC_{25} (nM)	EC_{50} (nM)	Concentration ^{T} (nM)	EROD		REP value ($EC_{5\ TCDD}$)	REP value (EC_{25})	REP value (EC_{50})
TCDD	$5*10^{-12}$	$8.9*10^{-12}$	$1.78*10^{-11}$	1	100	± 21 *	1	1	1
PCB-126	$5.6*10^{-11}$	$1.26*10^{-10}$	$2.11*10^{-10}$	5	79	± 4.1 *	0.1	0.03	0.08
				(µM)					
PCB-105	$2.50*10^{-6}$			10	19	± 0.8 *	0.000002		
PCB-118	$5.60*10^{-6}$			10	7	± 0.3 *	0.000001		
PCB-156	$1.10*10^{-7}$	$2.8*10^{-7}$	$3.2*10^{-7}$	5	68	± 3.2 *	0.000045	0.000150	0.000056
PCB-167					nd				

^{T} *concentration resulting in maximal induction*

** statistically significant compared to the vehicle control (DMSO 0.1%) ($p<0.05$)*

Table 2; Effects of mono-*ortho* PCBs, TCDD, and PCB-126 on AhR-EGFP expression and EROD activity in stably transfected rat hepatoma (H4G1.1c2) cells. The data are presented as one representative experiment (± SEM), conducted in triplicate.

Table 3

Effects of mono-ortho-PCBs in TCDD-induced AhR-expression and EROD activity in mouse H1G1.1c3 cells

Compound	Concentration † (µM)	EC_{25} (nM)	EC_{50} (nM)	AhR-EGFP			Compound	Concentration † (µM)	EC_{25} (nM)	EC_{50} (nM)	EROD		
PCB-105	1	$8.51*10^{-12}$	$2.24*10^{-11}$	58	± 4.5	•	PCB-105	1	$1.6*10^{-11}$	$4.0*10^{-11}$	57	± 4.8	•
	5	$1.12*10^{-11}$	$5.6*10^{-11}$	56	± 8.4	•		5	$1*10^{-11}$	$3.2*10^{-11}$	26.7	± 9.3	•
	10	$6.31*10^{-13}$	$3.55*10^{-11}$	50.9	± 2.9	•		10	$6.7*10^{-12}$	$3.5*10^{-11}$	39.3	± 2.8	•
PCB-118	1	$3.5*10^{-11}$	$4.5*10^{-11}$	73.7	± 2.2	•	PCB-118	1	$1.9*10^{-11}$	$4.2*10^{-11}$	82.8	± 3.9	
	5	$8.9*10^{-12}$	$3.55*10^{-11}$	42.3	± 11	•		5	$1.3*10^{-11}$	$3.8*10^{-11}$	46.5	± 10.4	•
	10	$5*10^{-12}$	$5*10^{-11}$	58.3	± 2.2	•		10	$1.1*10^{-11}$	$6.3*10^{-11}$	51.1	± 1.3	•
PCB-156	1	$1.3*10^{-11}$	$3.3*10^{-11}$	90.9	± 14.9		PCB-156	1	$1*10^{-12}$	$1.3*10^{-11}$	81.2	± 9.5	
	5	$3.2*10^{-11}$	$8.9*10^{-11}$	99.1	± 3.4			5	$2.0*10^{-11}$	$4.2*10^{-11}$	67	± 5.0	•
	10	$1.8*10^{-11}$	$4.5*10^{-11}$	70.8	± 1.3	•		10	$3.2*10^{-11}$	$7.1*10^{-11}$	48.3	± 4.0	•
PCB-167	0.1	$8.9*10^{-12}$	$2.5*10^{-11}$	97.7	± 8.4		PCB-167	0.1	$1.6*10^{-11}$	$3.8*10^{-11}$	77	± 12.7	
	1	$1.12*10^{-11}$	$3.55*10^{-11}$	87	± 12.2			1	$1.6*10^{-11}$	$4.0*10^{-11}$	74.8	± 4.8	•
	5	$1*10^{-11}$	$3.55*10^{-11}$	89.7	± 6.4			5	$1.6*10^{-11}$	$4.0*10^{-11}$	86.2	± 3.4	

Effects of mono-ortho-PCBs in TCDD-induced AhR-expression and EROD activity in rat H4G1.1c2 cells

Compound	Concentration † (µM)	EC_{25} (nM)	EC_{50} (nM)	AhR-EGFP			Compound	Concentration † (µM)	EC_{25} (nM)	EC_{50} (nM)	EROD		
PCB-105	1	$1*10^{-11}$	$3.2*10^{-11}$	87	± 10.4		PCB-105	1	$1.3*10^{-11}$	$2.7*10^{-11}$	97.0	± 14	
	5	$4.0*10^{-13}$	$4.5*10^{-11}$	51.8	± 16.9			5	$1.2*10^{-11}$	$3.9*10^{-11}$	68.0	± 12.2	
	10	$1.3*10^{-13}$	$2.1*10^{-11}$	76.0	± 7.2			10	$4.5*10^{-13}$	$3.2*10^{-11}$	96.0	± 5.3	
PCB-118	1	$7.1*10^{-12}$	$1.4*10^{-11}$	37.7	± 7.6	•	PCB-118	1	$1.1*10^{-11}$	$1.8*10^{-11}$	94.0	± 18	
	5	$2.8*10^{-12}$	$1.8*10^{-11}$	42.0	± 21			5	$1.1*10^{-11}$	$2.5*10^{-11}$	72.0	± 17.9	
	10	$6.7*10^{-12}$	$4.2*10^{-11}$	66.0	± 5.4			10	$1.2*10^{-11}$	$3.5*10^{-11}$	65.0	± 12.6	
PCB-156	1	$1.4*10^{-10}$	$1.6*10^{-10}$	54.2	± 7.8		PCB-156	1	$1*10^{-10}$	$1.5*10^{-10}$	105.0	± 17.3	
	5	$2.2*10^{-11}$	$1.3*10^{-10}$	75.7	± 4.2			5	$2.2*10^{-12}$	$1*10^{-10}$	129.0	± 20.1	
	10	$1*10^{-10}$	$2.0*10^{-10}$	64.8	± 1.3	•		10	$1*10^{-11}$	$5*10^{-11}$	85.0	± 3.7	
PCB-167	0.1	$5.6*10^{-12}$	$1.1*10^{-11}$	59.2	± 22.8		PCB-167	0.1	$7.9*10^{-12}$	$1.5*10^{-11}$	123.0	± 34	
	1	$5.6*10^{-12}$	$1.4*10^{-11}$	40.2	± 14.3	•		1	$7.9*10^{-12}$	$1.4*10^{-11}$	86.0	± 17.1	
	5	$5*10^{-12}$	$1.5*10^{-11}$	83.1	± 10.6			5	$7.6*10^{-11}$	$1.4*10^{-11}$	131.0	± 2.7	

† *concentration needed to obtain a maximum*

• *statistically significant compared to the positive control (TCDD 1 nM) ($p<0.05$)*

Table 3 (page 120); Effects of mono-*ortho* PCBs on TCDD-induced AhR-EGFP expression and TCDD-induced EROD activity in stably transfected mouse hepatoma (H1G1.1c3) and rat hepatoma (H4G1.1c2) cells. The data are presented as one representative experiment (± SEM), conducted in triplicate. •; statistically significant compared to the positive control (TCDD 1 nM) ($p<0.05$).

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CHAPTER SEVEN

General Discussion

1 Introduction

Past experiences of adverse effects of persistent and bioaccumulative chemicals on man and wildlife such as DDT and polychlorinated biphenyls (PCBs) have made regulatory authorities cautious when it comes to introducing new chemicals into the environment. Nevertheless, polybrominated diphenyl ethers (PBDEs) had been in use for decades before the first signs of accumulative behaviour of some lower congeners became obvious [1]. Research on PBDEs has so far mainly focussed on the lower brominated BDE-47 and -99 and BDE-153, since these congeners make up the highest concentrations of PBDEs found in wildlife and humans, and seem to be the biological and toxicological most active congeners. In this thesis, we have examined some aspects of the mechanism of action of several highly purified environmentally relevant PBDEs (BDE-47, -99, -100, -153, -154, -183). BDE-77 is not environmentally relevant but resembles PCB-77 due to its lack of *ortho*-bromine and two adjacent bromine atoms on both aromatic rings, and was therefore included. Deca-BDE (BDE-209) was also included in these studies but proved insoluble at the concentration range tested (1-10 µM). The main objective of this PhD study was to assess whether these specific PBDEs were able to elicit dioxin-like activities or cause interference with the aryl hydrocarbon receptor (AhR) pathway.

2 Dioxin-like responses

Dioxin-like compounds comprise a large group of widespread environmentally persistent pollutants, including some polychlorinated biphenyls (PCBs), dibenzofurans (PCDFs) and dibenzo-*p*-dioxins (PCDDs). These chemicals can exert a wide spectrum of biological and toxicological activities similar to that of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and it is generally accepted that most, if not all of these effects are mediated via the Ah receptor pathway [2,3]. Reported effects of dioxin-like compounds include reproductive and developmental toxicities, endocrine disruption, immunologic and adverse dermatologic effects, systemic effects such as liver disease, carcinogenesis and altered expression of Cytochrome P450 expression [3-7]. Some PCBs that have no or only one chlorine atom in the *ortho* position were found to have dioxin-like properties [3,8], which resulted in inclusion of these congeners in the toxic equivalence (TEQ) concept for dioxin-like compounds [2,9]. The structural similarities of certain PBDE congeners with PCBs have consequently raised concerns whether these brominated flame retardants

(BFRs) are also able to elicit dioxin-like responses. This concern was the major reason for studying this group of compounds in this PhD study.

2.1 Are PBDEs agonists of the Ah receptor?

Cytochrome P450 (CYP P450) enzymes are involved in the oxidative metabolism of structurally diverse xenobiotics, including steroid hormones, bile acids, fatty acids, and prostaglandins [10]. They also participate in the detoxification of carcinogens, environmental pollutants and drugs, by conversion to more polar molecules that are excreted more easily from the body [11]. Induction of the CYP1A1 enzyme is considered to be a sensitive indication of AhR binding and activation.

In previous studies, it has been reported that several PBDEs can competitively bind to the AhR and induce biological responses such as CYP1A1 mRNA and protein synthesis. In this respect 7-ethoxyresorufin-O-deethylase (EROD) activity as well as expression of an AhR-responsive luciferase reporter gene have been used to identify dioxin-like behavior [12-14]. No CYP1A1 induction using EROD activity as a marker was observed for BDE-47, -99, -154 in several cell culture systems [12]. However, a weak induction of CYP1A1 activity by BDE-77, -100, -153, -183 that was consistent with weak to medium binding to the dioxin-responsive elements (DRE) has also been observed [14]. The environmentally relevant BDE-47 and BDE-99 were among the least active, while the non-*ortho* substituted BDE-77 activated the AhR-DRE complex formation of rat cytosolic AhR at 60% of the maximal inducing TCDD concentration, and also induced CYP1A1 protein [14]. Similarly, using a genetically modified cell system (DR-CALUX assay), Behnisch and co-workers (2003) found that these PBDEs could weakly induce CYP1A1 (EROD) activity [13].

In this PhD study with these PBDEs (BDE-47, -77, -99, -100, -153, -154, -183, -209) no such induction of CYP1A1 mRNA or EROD was observed in the AhR responsive MCF-7 (human breast carcinoma), HepG2 (human hepatoma) and H4IIE (rat hepatoma) cell lines (Chapter one).

In commercial mixtures of PBDEs (Penta-BDE, Octa-BDE, Deca-BDE), low levels of contamination by polybrominated dibenzofurans and polybrominated dibenzo-*p*-dioxins (PBDF, PBDD) may occur [15]. These compounds bind to the AhR with higher affinity than could be expected from non-planar compounds like PBDEs, and consequently can induce AhR-dependent gene expression. When these impurities remain in the PBDE standards after appropriate clean-up, they may be responsible for part of the observed CYP1A1 induction in exposed cell systems that is otherwise attributed to PBDEs. This 'impurity' effect has also been seen by

Koistinen and co-workers (1996) when conducting assays with polychlorinated diphenyl ethers (PCDEs) [16]. These workers reported that even a small contamination with PCDFs (less than 1% by weight) almost entirely explained the observed induction that initially was attributed to these PCDEs.
In view of this impurity issue, the PBDE congeners that were used for our studies have been subjected to a specific purification on activated charcoal and Celite to remove possible contamination with dioxin-like compounds such as PBDFs [17]. When comparing the results presented in this PhD study with that of earlier studies, it most likely explains the differences in observed CYP1A1 induction by PBDEs reported by various laboratories.

Since primary cell cultures of hepatocytes are considered to be a more realistic and possible sensitive method to measure CYP1A(1) activity than (genetically modified) cell lines [14,18-20], the ability of PBDEs to induce CYP1A was assessed in primary cultures of cynomolgus monkey (*Macaca fascicularis*) hepatocytes as presented in chapter two [21]. Van der Burght *et al* demonstrated that several (multiple) *ortho*-substituted PCBs were able to induce CYP1A in hepatocytes from cynomolgus monkeys, although with weak potency [22]. In spite of this finding in this *in vitro* model, no significant induction of EROD activity by these PBDEs was observed in cynomolgus monkey hepatocytes. Even the non-*ortho* (lateral substituted) BDE-77 that structurally resembles the planar dioxin-like PCBs was not able to induce CYP1A in these hepatocytes. This lack of CYP1A induction of PBDEs in monkey hepatocytes further supports the earlier conclusion that PBDEs do not elicit AhR-mediated activity. With respect to the earlier results observed with multiple *ortho* substituted PCBs in these cynomolgus hepatocytes, it could be suggested to examine the possibility of an 'impurity' issue in more detail [22].
However, AhR activation by BDE-77, -100, -153, -183 has been reported previously in primary cell cultures of rat hepatocytes [12-14], although the observed induction of CYP1A1 was small and occurred only at very high concentrations. As with the multiple *ortho* substituted PCBs it is again suggested to examine a possible 'impurity' effect for this specific subset of PBDEs. With the exception of BDE-77, there seem to be no structural arguments [3] to support a possible AhR-mediated activation.
Clearly, there is a significant inconsistency with respect to the (lack of) AhR-mediated activities when evaluating the results of our experiments and earlier studies published in literature. Based on our results with different mammalian cell lines, we suggest that a rigid and sensitive clean-up procedure is necessary to

remove small amounts of AhR-active components to purify PBDEs properly before any studies should be conducted.

If we assume that the results of earlier studies were not influenced by the presence of more potent AhR agonist and the observed transcriptional effects of CYP1A1 were actually caused by the PBDEs, we can make an initial risk assessment as a kind of worst case approach. In the human body, European concentrations of PBDEs are currently maximally ~10 ng/g lipid weight (breast milk) [23-28]. The observed *in vitro* dioxin-like induction of CYP1A1 activity however occurs in the relatively high micromolar concentration range. This seems to be at least a thousand-fold higher than current environmental levels in biota, including humans.

2.2 Are PBDEs antagonists of the Ah receptor?

The PBDEs that were tested in mammalian cell lines (MCF-7, HepG2, and H4IIE) and primary cultures of cynomolgus monkey (*Macaca fascicularis*) (Chapter one and two) all showed a concentration-dependent antagonistic effect towards TCDD-induced CYP1A(1) induction using EROD activity as a marker. However, this antagonistic effect on enzyme activity could not be confirmed by measuring lower mRNA expression levels [29].

These antagonistic effects were less pronounced in the primary cynomolgus monkey hepatocytes [21]; the HepG2 cell line appeared to be the most sensitive model [29].

A similar antagonistic effect at the level of catalytic CYP1A1 activity was observed previously in cell lines and cultures of primary rat hepatocytes for PCB-153 [30] and for BDE-47, BDE-99 (strong) and BDE-77, -100, -126, -153, -156 (weak)[12,14]. In addition, Chen and Bunce (2003) also found that PBDEs did not change mRNA levels after co-exposure with TCDD even though an inhibitory effect was seen on EROD activity. Cytotoxicity and direct catalytic inhibition were not observed in our studies (Chapter one and two), and could therefore be excluded as a reason for this antagonistic effect.

One of the suggested mechanisms is that a low level of activated nuclear AhR by TCDD is enough to maintain the transcription of the CYP1A1 gene and would therefore not result in a difference in mRNA CYP1A1 level. This however does not explain the decreased EROD activity in our study and that of Chen and co-workers [14]. Another explanation might be that PBDEs interfere with other post transcriptional processes such as heme synthesis as previously suggested for PCB-77 [31]. It has also been suggested that polyhalogenated aromatic hydrocarbons

(PHAHs) are able to cause a dose-dependent increase in porphyrines [32], which are precursors of heme.

The fact that antagonism of PBDEs did not lead to a significant shift in EC_{50} values after co-exposure with TCDD (Chapter one and two)[21,29], but more clearly resulted in a decreased maximum in EROD activity, could originate from unproductive binding of these PBDEs to the AhR. This was suggested previously for PCB-153 [33]. If PBDEs bind the AhR unproductively, binding AhR by PBDEs would not directly affect the EROD activity since AhR binding alone is not sufficient to elicit a response [34-36].

This binding to the AhR would only have an implication on CYP1A mRNA and protein synthesis when all receptors are bound, e.g. at high concentrations of both the productive ligand (agonist) as well as the unproductive ligand (antagonist). It can be suggested that at lower concentrations of both the agonist as well as the antagonist, there would still be sufficient receptors available for the agonist to activate the signal transduction pathway and elicit a response (i.e. CYP1A1 activity) similar to that without an antagonist.

In addition, it should also be noted that the observed inhibitory effects by PBDEs were predominantly observed at relatively high levels of TCDD, which may have little relevance for the actual background exposure situation of the human population. Several critical arguments can be given when evaluating the possible relevance of these antagonistic effects for the human situation. The concentration range in which the individual PBDEs were showing antagonism in our experiments (1 µM tetrabromodiphenyl ether to 10 µM heptabromodiphenyl ether, corresponds with 0.5 µg/g up to 7.2 µg/g resp.) exceed the total PBDE concentration in human blood (approx. 3 ng/g lipid weight [37]), human breast milk (approx. 4 ng/g lipid weight [38]) and in human adipose tissue (approx. 12 ng/g lipid weight [39]) with many orders of magnitude. However, data from literature often represent the total PBDE (ΣPBDE) concentration, while we studied the interaction between TCDD and individual congeners. Summarizing, it can be concluded that the antagonistic effects between TCDD and PBDEs reported in our studies, probably do not have high relevance for the European human background situation due to significant higher levels used in our *in vitro* experiments when compared with human blood levels. However, it's relevance for the occupational and North American situation must be further examined.

3 Aryl hydrocarbon receptor pathway

It has been suggested that certain di-*ortho* substituted PCBs bind the AhR but do not cause CYP1A1 induction, depending on the chlorine substitution pattern [33,35,40]. This ligand binding to the AhR is probably unproductively as suggested previously for PCB-153 [33,35]. We hypothesized that the observed antagonistic behavior of PBDEs towards TCDD-induced CYP1A activity as observed in our experiments and earlier studies [14,21,29,41] might also be explained by such a mechanism analogue to di-*ortho* substituted PCBs.

To elucidate this interactive mechanism we performed experiments using the genetically modified mouse hepatoma (H1L1.1c2) and rat hepatoma (H4L1.1c4) cell lines containing a stable AhR-dependent enhanced green fluorescent protein (EGFP) construct (Chapter three). In both AhR-EGFP rodent cell lines, exposure to the purified PBDEs alone did not result in any AhR-mediated signals. However, co-exposure with TCDD again resulted in a significant decrease in TCDD-induced CYP1A1 (EROD) activity in these AhR-EGFP cell lines. For the lower brominated PBDEs, like BDE-47, this decrease in EROD activity concurred with a decrease in AhR-EGFP reporter gene expression. This concurrence between AhR-EGFP and CYP1A1 expression shows that this antagonistic effect between PBDEs and TCDD is caused by effects on AhR binding and activation rather than by simple competitive inhibition of the catalytic activity of CYP1A1 (See chapter three). Thus, these results indicate that PBDEs can bind the AhR as antagonists and compete with more toxic agonists e.g. TCDD for binding the AhR in analogy with what was found for some PCBs [34,42]. However, it was also observed that some of the higher brominated PBDEs like BDE-183 reduced TCDD induced CYP1A1 activity, while not influencing the AhR-EGFP gene expression. This might suggest that these PBDEs could reduce EROD activity by virtue of their ability to act as competitive CYP1A1 substrates as has been observed for other chemicals such as the PCBs [33].

Antagonistic effects by the higher chlorinated PBDEs were also observed at the level of the xenobiotic response element (XRE) in a stable transfected human hepatoma cell line (XRE-HepG2) as described in chapter three. Binding to the XREs on the DNA was indirectly assessed by the use of synthetic reporter genes, which contain XRE binding sites in a heterologous promotor context that report the increased basal activity [43 559]. All PBDEs tested in our study elicited an inhibitory effect on the TCDD-induced XRE-driven luciferase activity. However, based on our data it seems that the affinity of the higher brominated congeners (e.g. BDE-183) is lower than that of the lower brominated congeners (e.g. BDE-99) with regard to the

AhR-XRE mediated effects. Binding to the XRE on DNA as indirectly measured in our experiments, suggests that some of these PBDEs are capable of initiating the AhR transformation as well as the nuclear localization.
A further analysis of the effects of BDE-99, the congener giving the most consistent and pronounced inhibitory effects on luciferase driven XRE activity in the HepG2 cell line, was performed with the Gal4-AhR construct. Singular exposure to BDE-99 did not lead to significant effects on the AhR, but a Chromatin Immunoprecipitation Assay (ChIP) confirmed the ability of BDE-99 (10 µM) to allow the AhR to bind to the promotor region on the DNA (Chapter three).

4 Phenobarbital-like responses

Phenobarbital (PB)-like responses include alteration of expression of several CYP P450 enzymes, including CYP2B and CYP3A. CYP3A4 is of particular significance in humans because this enzyme is involved in the metabolism of a large number of clinically used drugs [11]. Adverse PB-like effects of non dioxin-like PCBs are among others altered thyroid hormone homeostasis, neurodevelopmental effects and tumor promotion [44-46].
PBDEs have been reported to elicit similar effects on the thyroid hormone homeostasis in *in vivo* and *in vitro* studies [47-50] and to elicit neurobehaverioral effects, among others [51-54].
Previous findings and those presented in this thesis that PBDEs were able to antagonize TCDD-induced responses and bind the AhR unproductively in analogy with e.g. PCB-153 [21,29] lead to the hypothesis that these PBDEs might be able to elicit PB-like responses as multiple *ortho* substituted PCBs do [30,33,42].
Sanders and co-workers (2005) reported that BDE-47, -99, and BDE-153 were able to upregulate *CYP3A* as well as *CYP2B* gene expression in rats. Obviously, there are large species differences in *CYP3A4* and the associated human pregnane X receptor (hPXR) protein. Some of these can be explained by sequence differences in the ligand-binding domain of the PXR, and not at the DNA binding site [55,56]. In the case of the *CYP3A4* gene this makes extrapolation of animal models to the human situation more difficult [57-59].
In transiently transfected human hepatoma (HepG2) cells with the hPXR and *hCYP3A4*, the lower brominated PBDEs (BDE-47, -77, -99, -153) were able to induce *CYP3A4* expression. This expression coincided with activation of the hPXR. Surprisingly, exposure of the transiently transfected HepG2 cells to TCDD also resulted in an increase in *CYP3A4* expression and hPXR activation, but we were

not able to confirm this with quantitative RT-PCR. Results of these experiments are presented in chapter four of this thesis. The fact that dioxin-like chemicals are able to induce PB-like responses like *CYP3A4* gene expression seems to indicate that there exist some kind of interaction between the AhR and hPXR as proposed recently by Shaban and co-workers (2005) [60].
Interference of xenobiotics with CYP3A4 could have implications for drug-drug interactions as CYP3A4 is the predominant drug metabolizing enzyme in the human liver [61]. However, the observed *in vitro* effects by PBDEs occurred at medium concentrations that usually exceed the average European human blood levels. Assuming that *in vitro* medium concentrations (approx 10 µM) could be used as a first approach for human blood or plasma levels it is not expected that levels in humans would be high enough to initiate a possible CYP3A4 interaction by PBDEs.

5 Legislation

Current legislation is not adequate to avoid the entrance of bioaccumulative chemicals into the environment however; a start has been made with the introduction of REACH (the Registration, Evaluation and Authorization of Chemicals) in the European Union. This regulation should provide a strong safety net before chemicals are allowed on the European market in the future.
For PBDEs, the current situation is that the European Union Community banned the use of Penta-BDE and Octa-BDE since July 31 of 2004[6]. This was followed by the sole US producer (Great Lakes Chemical Corporation, IN, USA) that voluntarily ceased production of these two PBDE mixtures by the end of 2004. The current end of production of these two commercial PBDE mixtures should lead to a decline in biota and abiota of these chemicals. Such a decline as has already been reported in human breast milk samples from Sweden for the lower brominated PBDEs [62]. However, due to the persistent nature of some PBDEs, it could take decades before this decline can be observed in all environmental matrices worldwide as there will also be continuing exposure through older end-product use. The continued use of Deca-BDE is still under discussion within the United

[6] European Union, Restriction of Hazardous Substances Directive (RoHS); Directive 2002/95/EC of the European Parliament and of the Council of 27 January 2003 on the restriction of the use of certain hazardous substances in electrical and electronic equipment, OJ L37, 13 February 2003, page 19

States, but its use is currently not restricted in the European Union[7]. Deca-BDE is the most commonly used commercial PBDE flame retardant, but BDE-209 is not the main PBDE congener found in human samples [63]. The continued use of Deca-BDE has lead to concerns about the possible debromination of the deca-substituted congeners into lower brominated and possibly more active congeners such as BDE-47 and -99 [64]. Metabolism and UV-light are the proposed pathways for debromination [65,66], but at present no evidence is available that the debromination of the Deca-BDE is the major source of lower brominated BDEs in the environment and biota.

There are many alternatives to PBDEs; not only other BFRs, but also other, non-brominated flame retardants. Currently 39% of the worldwide flame retardant market consists of BFRs [67], at present PBDEs are by far the most studied flame retardants all over the world. Replacing e.g. Deca-BDE with another less well studied (non-brominated) flame retardant chemical could also pose a risk for man and his environment. Yet unknown hazards might exist for less well studied replacements of existing brominated flame retardant. In addition, the benefits of fire safety might be not as adequate for less studied flame retardants. When discussed within the scientific community whether Deca-BDE should be replaced, it should be evaluated if possible replacement(s) have been equally well studied as the PBDEs, in particular Deca-BDE, with respect to actual internal exposure and possible adverse effects.

Clearly, the discovery of some PBDEs in e.g. human breast milk could be an alarming finding and the concentrations are high enough to expect an effect based on experimental studies. However, their mere presence alone should not be the reason for concern as with our advanced analytical techniques we can nowadays detect almost every compound in humans with which we are in contact with. In addition, we should not forget that living the current modern life style means that we are in contact with many potential hazardous compounds, but that it is the actual dose (and time) that makes the poison. Therefore, future legislation will not be able to remove all these possible risks from our lives, but we can certainly try to replace potential toxic chemicals by lesser hazardous substances. If singular compounds do not show any toxicological activities at relevant exposure levels,

[7] Commission Decision of 13 October 2005 amending for the purposes of adapting to the technical progress the Annex to Directive 2002/95/EC of the European Parliament and of the Council on the restriction of the use of certain hazardous substances in electrical and electronic equipment, OJ L271, 15 October 2005, page 48

such as the inability of PBDEs to activate the AhR mediated (dioxin-like) effects as shown in this PhD study, this does not mean that they do not pose a threat through other mechanisms of action. In this respect the interaction of PBDE metabolites with e.g. the thyroid hormone homeostasis and estrogen receptor or the neurotoxic effects of these compounds clearly deserves more attention. In addition, humans and wildlife are exposed to a wide array of chemical compounds that can cause interactive effects with combinations of dioxins and PBDEs as was shown by our studies. Therefore, it should also be mentioned that complex mixture effects should not be ignored in future risk assessments, which should not be based on the dose-effect relationship of the individual compounds only. Such an effect of complex mixtures does not always have to be additive or synergistic, but can also be antagonistic with possible reduction in biological or toxicological activity. This was illustrated by the results of the experiments presented in this thesis using combinations of TCDD and various PBDEs, although a first assessment indicates that it might not be highly relevant for the human exposure situation.
Currently, PBDEs have not been assigned toxic equivalency factors (TEF) for dioxin-like compounds like certain non- or mono-*ortho* PCBs (Chapter five) [2]. If a compound does not bind to and activate the AhR, the compound need not be assigned a TEF value [2,68]. The absence of CYP1A1 induction by the environmentally relevant PBDEs tested in our study supports the increasing body of evidence that these compounds do not require inclusion in the TEF concept for dioxin-like compounds.

6 In conclusion

The results of our studies presented in this thesis indicate that PBDEs do interact with the mammalian AhR and PXR receptor transduction pathways. The PBDEs used in our study were not able to activate the AhR, and consequently not able to induce CYP1A1 enzyme activity and/or CYP1A1 mRNA neither in the human MCF-7 and HepG2 and rodent H4IIE cancer cell lines nor in primary monkey (*Macaca fascicularis*) hepatocytes. Based on these results we see no argument to support inclusion of these compounds in the TEF concept for dioxin-like compounds. Further research is necessary to assess the full scope of possible toxic effects of PBDEs since there is evidence that they may act as PB-like compounds as indicated by the interaction with the hPXR receptor and *CYP3A4* gene induction.
In addition, a dose-dependent antagonistic effect of PBDEs was observed on TCDD-induced CYP1A, measured as EROD activity. These antagonistic effects

were seen at PBDE concentrations ranging from 0.1 to 10 µM, while individual levels of PBDE in humans are estimated to be generally lower than 1 nM in the background situation in Europe [69]. This antagonistic effect was seen at relative high levels of induction of CYP1A by TCDD that are not easily expected in the European human background population. Thus, the observed antagonistic effects of PBDEs on TCDD-induced biological activity in our experiments probably have little or no relevance for the human situation. However, recently it has been demonstrated that levels in the United States can be significantly higher than in Europe. Further (*in vivo*) studies may be necessary for confirmation.

The mechanism of action of lower brominated PBDEs seems to differ from higher brominated congeners. The mechanism for this is unknown, but as BDE-99 can cause nuclear translocation of the AhR without initiation of transcriptional activation, this compound could cause a unfavorable conformational change of the receptor, which does not allow binding to co-factors and/or components of the initiation complex. Observed interactions for some of the higher brominated PBDEs on CYP1A1 activity may be AhR-independent and likely occur by competitive inhibition of CYP1A1-dependent EROD activity, but this remains to be confirmed.

The observed effects might have implications for risk assessment as humans are exposed to a complex mixture with a large number of dioxin-like compounds, PBDEs and PCBs. As many quantitatively important PBDEs and PCBs can apparently act as AhR antagonists and most likely act in concert when present in mixtures, this could actually influence the overall effect of dioxin-like compounds in a down-regulating way. Based on our *in vitro* results the question remains to which extent these PBDEs could actually inhibit or antagonize AhR-mediated toxicological and biological effects *in vivo*, which should clearly be assessed further.

The combined results of the described experiments imply that the PBDEs do not need to be assigned TEF values for dioxin-like compounds. On the basis of these results, among others, the World Health Organisation (WHO) has decided at a dioxin and PCB expert meeting in June 2005 to exclude these PBDEs from the risk evaluation of dioxin-like compounds in the TEF system [70].

What aggravates the current understanding of the toxicity of PBDEs is the possibility of impurities in the test compounds. The need to purify PBDEs has been reported before [29,71,72] and further supported by the experiments done for this PhD project. Small impurities of the test compounds with AhR-active compounds could

disturb the outcome of the experiments [16,73]. We have studied this further with the use of certain mono-*ortho* substituted PCBs (PCB-105, -118, -156, -167). These compounds were also subjected to a rigorous clean-up procedure on active charcoal as described for the PBDEs used in our studies (Chapter six). Using this clean-up procedure for the MO-PCBs resulted in remarkably low REP values for these compounds, which were significantly lower, ~15 up to maximally ~125 fold, than the TEF values proposed by the WHO [2].

In addition, we suggest that the observed differences in dioxin-like effects of PBDEs between the results presented in this thesis and earlier publications were not primarily due to species differences and different effect parameters, but could more likely be caused by small impurities in the test compounds as previously observed for PBDEs [71], PCDEs [16], and PCBs [73]. Thus, it is highly important to rule out the absence of impurities like dibenzofurans and dioxins when studying biological or toxicological properties of individual PBDEs or their commercial mixtures by applying rigorous clean-up procedures.

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Gebromeerde Diphenyl Ethers

Enkele aspecten van het werkingsmechanisme

Nederlandse Samenvatting

Gebromeerde diphenyl ethers

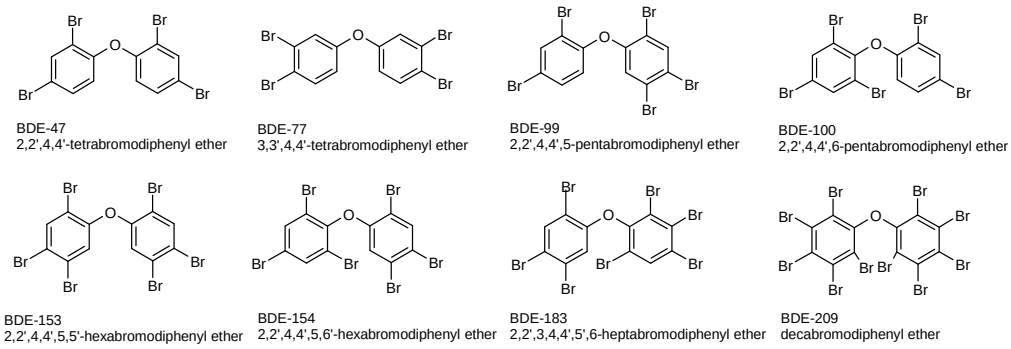
Vlamvertragers zijn sinds de jaren zeventig veelvuldig gebruikt als brandvertragende toevoegingen aan bijvoorbeeld plastics van computers, tv's, isolatiemateriaal in kabels, maar ook in kussens en matrassen. Dit heeft als gevolg dat wij in ons dagelijks leven met grote regelmaat worden blootgesteld aan deze stoffen.

Vlamvertragers kunnen stikstof bevattend, fosfor bevattend, anorganisch, gechloreerd, of gebromeerd zijn. De groep van gebromeerde vlamvertragers vertegenwoordigt wereldwijd 39% van alle brandvertragende stoffen en wordt als de meest effectieve beschouwd wanneer men naar kosten en effectiviteit kijkt. De werkzaamheid berust op het afsplitsen van het halogeenatoom bij hogere temperaturen.

De groep van gebromeerde vlamvertragende stoffen kan verder onderverdeeld worden in een aantal commerciële mengsels; tetrabromobisphenol A (TBBPA), hexabromocyclododecane (HBCD), Penta-BDE, Octa-BDE en Deca-BDE. De laatste drie zijn gebromeerde diphenyl ethers (PBDEs). PBDEs worden tegenwoordig wereldwijd gevonden in sediment, vetweefsel van vissen, wilde dieren en ook mensen. De detectie van PBDEs in humaan weefsel, bloedmonsters en moedermelk heeft tot veel vragen geleid omtrent mogelijke schadelijke effecten.

Net als PCBs worden PBDEs vernoemd naar de positie en het aantal atomen aan de phenylring, wat leidt tot 209 mogelijke congeneren. Het zijn gebromeerde organohalogeen verbindingen die een structurele overeenkomst hebben met persistente organohalogeen verbindingen (POPs). Met name de structurele overeenkomst met gechloreerde biphenyls (PCBs) heeft tot vragen geleid omtrent het werkingsmechanisme van PBDEs.

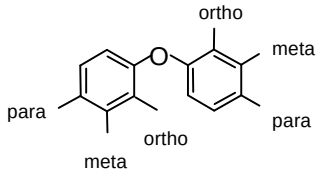
PCBs zijn, net als PBDEs, goed oplosbaar in vet en kunnen bioaccumuleren in het milieu: de stof stapelt zich op in de voedselketen doordat een roofdier naast zijn eigen belasting aan chemische stoffen ook dat van de prooi tot zich neemt.
Dit is duidelijk geworden na een ongeluk in de jaren zeventig in Michigan met polybroombiphenyl (PBB), een stof sterk verwant aan PCBs. Nadat veevoeder in contact was geraakt met PBBs, raakte naast de veestapel ook consumenten, de mens, besmet met PBBs. PCBs zijn in het verleden onder andere gebruikt in isolatievloeistof, koelvloeistof en als weekmaker in kunststof. De productie en het gebruik van PCBs zijn sinds 1985 verboden, maar concentraties van PCBs in het milieu zijn nog steeds meetbaar door het gebruik van PCB-bevattende materialen, aanwezigheid op vuilstortplaatsen en vuilverbranding. Het wordt algemeen aangenomen dat mensen voornamelijk via de voeding aan PCBs worden blootgesteld. Verondersteld wordt, dat voeding ook de voornaamste bron van blootstelling aan PBDEs is.



Figuur 1; de structuur van de PBDEs in dit onderzoek

In dit proefschrift hebben wij naar enkele werkingsmechanismen van sterk gezuiverde PBDEs gekeken. Een groot deel van het onderzoek was gericht op de vraag of PBDEs in staat zijn dioxine-achtige effecten te veroorzaken door middel van interactie met de aryl hydrocarbon receptor (AhR). Hierbij is gekozen voor de congeneren die het meest in het milieu voorkomen: BDE-47, -99, -100, 153, -154 en -183 (Figuur 1). Daarnaast is gekozen om ook BDE-77 nader te beschouwen. Dit, omdat BDE-77 qua structuur enige gelijkenis heeft met bepaalde PCBs die een dioxine-achtig werkingsmechanisme hebben zoals PCB-77. BDE-77 wijkt dan ook af van de andere bestudeerde PBDEs door het feit dat er geen broom atomen op de *ortho* positie van het molecuul zitten (Figuur 2). Deca-BDE (BDE-209) was in eerste

instantie ook inbegrepen, maar deze stof bleek onoplosbaar bij de gebruikte testconcentraties (0.1-10 µM).



Figuur 2; de structuur van de PBDEs

Dioxine-achtige toxiciteit

Van enkele planaire PCBs, bijvoorbeeld PCB-77, is bekend dat zij in staat zijn de aryl hydrocarbon receptor (AhR) te binden. Dit receptor eiwit bevindt zich in de cel en wordt na binding aan een ligand, naar de kern van de cel getransporteerd. Daar bindt de AhR aan een kern eiwit, de ARNT (Ah receptor nucleaire translocator). Het gevormde AhR:ARNT complex zal vervolgens binden aan bepaalde delen van het DNA, de XREs (xenobiotic response elements). Deze binding leidt tot transcriptie van een aantal genen, zoals bijvoorbeeld *CYP1A1.* Het *CYP1A1* gen codeert voor het CYP1A1 enzym, dat in staat is om lichaamsvreemde stoffen te metaboliseren tot moleculen die beter oplosbaar zijn en beter kunnen worden uitgescheiden. Sterke binding aan de AhR is een van de sleuteleigenschappen van dioxine-achtige stoffen, waarvan TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) de meest kenmerkende en potente congeneer is.

Om uit te zoeken of PBDEs (Figuur 1) in staat zijn om aan de AhR te binden en tot gen expressie leiden, is de inductie van het CYP1A1 enzym bestudeerd in een aantal verschillende zoogdier cellijnen, inclusief die van de mens. Een cellijn is een groep van cellen, vaak verkregen uit een tumor, die allen dezelfde eigenschappen hebben en onsterfelijk zijn; ze blijven continu delen. Dit maakt het mogelijk om ze voor veel soorten *in vitro* experimenten te gebruiken.

In dit onderzoek werd zowel de activiteit van het CYP1A1 enzym gemeten, alsmede het messenger RNA (mRNA). Vorming van een eiwit geschiedt doordat er transcriptie van het DNA optreedt, het mRNA dat op deze wijze gevormd wordt, kan vervolgens 'vertaald' worden naar een keten van aminozuren, het eiwit. Detectie van mRNA kan met behulp van reverse transcriptase polymerase chain reaction (RT-PCR). Detectie van een enzymactiviteit wordt meestal gedaan door de

omzettingssnelheid te meten van een geschikt substraat, zoals bijvoorbeeld ethoxyresorufine bij CYP1A1 in ons onderzoek.
Na blootstelling van humane borstkanker cellen (MCF-7), humane levercellen (HepG2) en rat levercellen (H4IIE) bleek dat deze PBDEs niet in staat waren zelf CYP1A1 te induceren, met andere woorden: de gekozen PBDEs bleken niet in staat tot activatie (agonisme) van het receptor complex. Dit gold zelfs voor de hypothetisch dioxine-achtige BDE-77.
Echter, nadat deze cellen gelijktijdig werden blootgesteld aan zowel TCDD als een PBDE, bleek dat deze gebromeerde vlamvertragers in staat waren om de werking van TCDD te verminderen (inhibitie of antagonisme). Bij deze mengsel-experimenten werd minder CYP1A1 gevormd dan door blootstelling aan TCDD alleen verwacht werd.

Aangezien cellen die direct uit een lichaam komen een betere representatie kunnen geven van wat er *in vivo* gebeurt en apen nauw verwant zijn aan de mens, is er gekozen om de voorgaande experimenten te herhalen in gezonde levercellen van cynomolgus apen (*Macaca fascicularis*). Zoals eerder gezien in de zoogdier cellijnen, bleek ook uit deze experimenten dat de PBDEs niet in staat waren tot inductie van CYP1A. Blootstelling aan zowel TCDD als PBDEs leidde wederom tot een daling van de inductie van CYP1A door TCDD.
Het feit dat de PBDEs in staat bleken om de inductie van CYP1A1 door TCDD te verminderen kan verschillende oorzaken hebben. Enkele voorbeelden: PBDEs zouden kunnen binden aan het CYP1A1 enzym waardoor de katalytische activiteit niet meer meetbaar is (katalytische inhibitie), of PBDEs zouden aan de Ah receptor kunnen binden zonder activatie van transcriptie processen waardoor TCDD niet meer beschikbaar is voor binding aan de AhR (antagonisme).
Om na te gaan of de geobserveerde inhibitie veroorzaakt werd door katalytische inhibitie of door antagonisme van de AhR, werden wederom zoogdier cellijnen (H1G1.1c3 muis en H4G1.1c2 rat hepatoma cellen) blootgesteld aan PBDEs en TCDD. De gebruikte cellijnen zijn moleculair getransfecteerd; een DNA construct (XRE) van de AhR gekoppeld aan een lichtgevend signaal (luciferase) is permanent in de cellen ingebouwd. Als stoffen aan de AhR binden en activatie van het XRE in het DNA optreedt, kan het luciferase signaal gemeten worden. Aanvullend werd in deze twee genetisch gemodificeerde cellijnen tevens de activiteit van het CYP1A1 enzym gemeten als maat voor de genexpressie. In aanvulling op deze experimenten werden humane levercellen (HepG2) getransfecteerd met een XRE, gekoppeld aan luciferase. Uit deze experimenten bleek dat de lager gebromeerde

PBDEs wel in staat waren om de XRE te binden, maar niet tot het activeren van de AhR. De inhibitie die eerder geobserveerd werd na gezamenlijke blootstelling werd wederom gemeten en bleek dus ook meetbaar op het niveau van de receptor. De effecten waren sterker voor de lager gebromeerde PBDEs (BDE-47, -77, -99). Hoger gebromeerde PBDEs zoals BDE-183 bleken wel in staat tot inhibitie van het CYP1A1 enzym na inductie door TCDD, maar hadden niet of nauwelijks effect op receptor niveau.
De resultaten uit deze verschillende experimenten tonen aan dat de sterk gezuiverde PBDEs wel in staat zijn te binden aan de aryl hydrocarbon receptor, maar niet in staat zijn tot genexpressie zoals inductie van het CYP1A1.
Deze gezamenlijke resultaten betekenen dat de PBDEs niet opgenomen hoeven te worden in het TEF (toxic equivalency factor) systeem, dat gebruikt wordt voor dioxine-achtige stoffen. Mede op grond van dit onderzoek heeft de Wereld Gezondheids Organisatie in een bijeenkomst van dioxine en PCB deskundigen in juni 2005 besloten om deze PBDEs verder uit te sluiten bij de risicoschatting van dioxine-achtige stoffen in het TEF systeem.

Phenobarbital-achtige toxiciteit

Het gebrek aan dioxine-achtige toxiciteit van PBDEs betekent niet dat deze stoffen in het geheel niet schadelijk kunnen zijn voor mens en dier. Enkele gerapporteerde effecten van PBDEs in proefdieren zijn verstoring van de schildklier hormoonhuishouding, verminderd geheugen en leercapaciteit. Deze effecten lijken sterk op de effecten van phenobarbital (PB). PB is een barbituraat en veel van de effecten worden veroorzaakt middels de activatie van de pregnane X receptor (PXR). Activatie van de PXR leidt tot inductie van Cytochroom P450 3A4 (CYP3A4), een enzym dat in de humane lever onder andere verantwoordelijk is voor de omzetting van geneesmiddelen in hun werkzame vorm. Om nader te onderzoeken of de gekozen PBDEs wellicht een PB-achtige werking hebben, is gebruik gemaakt van humane levercellen die getransfecteerd zijn met het *CYP3A4* gen en de humane PXR, gekoppeld aan een luciferase signaal. Naast het meten van luminescentie werd tevens onderzocht of op mRNA niveau effecten optraden. Uit de experimenten met deze getransfecteerde cellen bleek dat zowel PBDEs als TCDD in staat waren om *CYP3A4* te induceren, wat gepaard ging met activatie van de hPXR. Dit kon echter niet bevestigd worden op mRNA niveau. De resultaten zijn een eerste indicatie dat PBDEs mogelijk wel een effect zouden kunnen hebben

op de inductie van CYP3A4, maar verder onderzocht moet worden of dit ook daadwerkelijk *in vivo* optreedt bij relevante concentraties.

Zuivering van teststoffen

De teststoffen gebruikt in onze onderzoeken zijn allen sterk gezuiverd, om te voorkomen dat vervuilingen de uitkomst van de experimenten beïnvloeden. In het verleden zijn verschillende effecten van PBDEs gerapporteerd, waaronder dioxine-achtige effecten. Het feit dat wij in verschillende *in vitro* zoogdiersystemen geen significante CYP1A1 inductie hebben aangetoond komt waarschijnlijk door de rigoureuze zuivering van dioxine-achtige vervuilingen die deze stoffen hebben ondergaan. Een vergelijkbare discussie omtrent de rol van dioxine-achtige vervuilingen heeft plaatsgevonden omtrent de AhR gemedieerde activiteiten van mono-*ortho* gesubstitueerde PCBs. Om dit nader te onderzoeken hebben wij in de eerder genoemde getransfecteerde muis en rat levercellen enkele van de meest milieu relevante mono-*ortho* gesubstitueerde PCBs getest op een vergelijkbare wijze als de PBDEs. Deze mono-*ortho* PCBs zijn in tegenstelling tot de PBDEs wel opgenomen in het TEF systeem voor dioxine-achtige stoffen en in het verleden veelvuldig getest. Na een rigoureuze zuivering bleek dat deze PCBs een veel lagere AhR binding en inductie van CYP1A1 vertoonden dan uit bestaande literatuur kan worden opgemaakt. Voor zowel de geteste PBDEs als mono-*ortho* PCBs geldt dus dat een grondige zuivering van teststoffen een vereiste is voor betrouwbare uitkomsten van een experiment. Uit de eerdere literatuur kan helaas niet worden afgeleid of deze geteste PBDEs en mono-*ortho* PCBs voldoende gezuiverd waren van dioxine-achtige verbindingen. Gezien de gerapporteerde AhR gemedieerde effecten van beiden groepen stoffen in de literatuur moet op grond van dit promotie onderzoek gevreesd worden, dat een deel van de oudere resultaten beïnvloed zou kunnen zijn door de aanwezigheid van dioxine-achtige onzuiverheden in de testverbindingen.

Wetgeving

Ervaringen uit het verleden met persistente en bioaccumulerende chemicaliën zoals PBBs en PCBs, hebben er voor gezorgd dat men waakzaam is bij het introduceren van nieuwe chemicaliën. Desalniettemin heeft het enkele tientallen jaren geduurd voordat men er achter kwam dat enkele, met name lager gebromeerde, PBDEs in staat zijn om te bioaccumuleren.

De Europese Unie heeft daarom besloten dat de commerciële PBDE mengsels Penta-BDE en Octa-BDE van de markt gehaald moeten worden per augustus 2004 [8]. Dit besluit is vrijwillig gevolgd door de enige producent van deze mengsels in de Verenigde Staten. Dit zou ertoe moeten leiden dat deze stoffen langzaam verdwijnen uit het milieu. Er is reeds een daling van de concentraties gemeten in moedermelk monsters in Zweden ten opzichte van een aantal jaren geleden. Doordat PBDEs moeilijk afbreekbaar zijn en het gebruik van Penta- en Octa-BDE houdende materialen, vuilstort en vuilverbranding de komende jaren nog zal voortduren, blijven deze stoffen voorlopig nog wel meetbaar in de mens en zijn voedselketen.
De Deca-BDE mix is niet verboden in Europa [9], sterker nog, dit mengsel wordt als veilig gezien en het gebruik hiervan is recent door de EU goedgekeurd. In de Verenigde Staten is er nog steeds onderzoek gaande naar deze vlamvertrager en moet hierover nog een beslissing worden genomen.
Op het moment is Deca-BDE de meest gebruikte gebromeerde vlamvertrager. Angst bestaat dat deze vlamvertrager onder invloed van UV-licht of door metabolisme kan afbreken tot lager gebromeerde, mogelijk meer toxicologisch actieve congeneren zoals BDE-47 and -99, maar hiervoor is geen eenduidig bewijs. Hierbij dient te worden opgemerkt dat detectie van PBDEs in de mens op zich niet voldoende is om groot alarm te slaan, aangezien de dosis bepaalt of de stof een gif is.

[8] European Union, Restriction of Hazardous Substances Directive (RoHS); Directive 2002/95/EC of the European Parliament and of the Council of 27 January 2003 on the restriction of the use of certain hazardous substances in electrical and electronic equipment, OJ L37, 13 February 2003, page 19

[9] Commission Decision of 13 October 2005 amending for the purposes of adapting to the technical progress the Annex to Directive 2002/95/EC of the European Parliament and of the Council on the restriction of the use of certain hazardous substances in electrical and electronic equipment, OJ L271, page 48, 15 October 2005

Conclusie

Resultaten in dit proefschrift geven aan dat enkele PBDEs in staat zijn een interactie aan te gaan met de zoogdier Ah receptor en humane PX receptor. Interactie met de AhR leidt echter niet tot transcriptie van mRNA en enzym synthese van CYP1A1, een van de meest gevoelige effecten van dioxine-achtige stoffen. Derhalve hoeven PBDEs niet te worden opgenomen in het TEF systeem voor dioxine-achtige stoffen. Wel is verder onderzoek naar de mogelijke *in vivo* werking via de PX receptor wenselijk, waarbij ook de mate van blootstelling in overweging moet worden genomen. Het is mogelijk dat PBDEs een werkingsmechanisme zouden kunnen hebben dat ten dele overeenkomt met PB.
Aangezien de testconcentraties in onze experimenten hoger zijn dan de huidige achtergrondconcentratie in de mens (< 1 nM in Europa), heeft de aangetoonde inhibitie van TCDD geïnduceerde effecten waarschijnlijk weinig of geen gevolgen voor de mens. Daarnaast zijn onze experimenten uitgevoerd in een *in vitro* situatie; het is de vraag of de effecten geobserveerd in deze gecontroleerde laboratoriumsituatie ook daadwerkelijk in een intact levend lichaam (*in vivo)* kunnen worden waargenomen.

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Curriculum Vitae

Annelieke Katrien Peters was born, in Eindhoven, The Netherlands, on the 30th of June 1978. In 1996, she graduated from the Sondervick College in Veldhoven. In the same year, she expanded her subjects at high school at the Regionaal Opleidings Centrum (ROC) in Eindhoven, which was rewarded with a diploma. She began her study Biomedical Health Sciences at the Catholic University in Nijmegen in 1997. For her minor 'Pharmaceutical Research' she studied the detectability of positive and negative strand hepatitis C virus in sanctuary sites at the Eijkman Winkler Institute (University Medical Centre, Utrecht) under the supervision of Dr. C. Visser and Prof. Dr. F.G.M. Russel. For her major 'Toxicology' she investigated the mutagenicity of nutricional supplements at Royal Numico Research BV (Wageningen), under the supervision of Dr. I.T.M. Becks-Vermeer and Dr. R.P. Bos. She received her Masters degree in 2001, and continued in the field of toxicology with her Ph. D. project at the Institute for Risk Assessment Sciences (IRAS; Utrecht University) in 2002. The Ph. D. project focused on aspects of the mechanism of action of polybrominated diphenyl ethers (PBDEs). A small part of this project was performed at the research group of Prof. Dr. D. Schrenk (University of Kaiserslautern, Germany) and with the research group of Prof. Dr. L. Poellinger (University of Stockholm, Sweden).