TOXICOLOGICAL SCIENCES **92(1)**, 157–173 (2006)
doi:10.1093/toxsci/kfj187
Advance Access publication April 6, 2006

In Vitro Profiling of the Endocrine-Disrupting Potency of Brominated Flame Retardants

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Received February 1, 2006; accepted April 1, 2006

Over the last few years, increasing evidence has become available that some brominated flame retardants (BFRs) may have endocrine-disrupting (ED) potencies. The goal of the current study was to perform a systematic *in vitro* screening of the ED potencies of BFRs (1) to elucidate possible modes of action of BFRs in man and wildlife and (2) to classify BFRs with similar profiles of ED potencies. A test set of 27 individual BFRs were selected, consisting of 19 polybrominated diphenyl ether congeners, tetrabromobisphenol-A, hexabromocyclododecane, 2,4,6-tribromophenol, *ortho*-hydroxylated brominated diphenyl ether 47, and tetrabromobisphenol-A–bis(2,3)dibromopropyl ether. All BFRs were tested for their potency to interact with the arylhydrocarbon receptor, androgen receptor (AR), progesterone receptor (PR), and estrogen receptor. In addition, all BFRs were tested for their potency to inhibit estradiol (sulfation by estradiol sulfotransferase (E2SULT), to interfere with thyroid hormone 3,3′,5-triiodothyronine (T3)–mediated cell proliferation, and to compete with T3-precursor thyroxine for binding to the plasma transport protein transthyretin (TTR). The results of the *in vitro* screening indicated that BFRs have ED potencies, some of which had not or only marginally been described before (AR antagonism, PR antagonism, E2SULT inhibition, and potentiation of T3-mediated effects). For some BFRs, the potency to induce AR antagonism, E2SULT inhibition, and TTR competition was higher than for natural ligands or clinical drugs used as positive controls. Based on their similarity in ED profiles, BFRs were classified into five different clusters. These findings support further investigation of the potential ED effects of these environmentally relevant BFRs in man and wildlife.

Key Words: **toxicity profiling; brominated flame retardants; endocrine disruption; hierarchical cluster analysis; principal component analysis.**

Problem

Over the past 50 years, petroleum-based polymeric materials have increasingly been used in construction materials, textiles, and computer equipment. To meet fire safety regulations for consumers of these products, flame retardants are incorporated in these inflammable polymers to increase their fire resistance. About 25% of the annual flame retardant production consists of halogenated organics, of which brominated flame retardants (BFRs) are the most important (Alaee *et al.*, 2003). Although BFRs are beneficial to mankind due to their flame-retarding characteristics, some may also pose a health risk to man and the environment, given their persistent, bioaccumulating, and suspected endocrine-disrupting (ED) potencies.

Characteristics of BFRs

Of all BFRs, tetrabromobisphenol-A (TBBPA), polybrominated diphenyl ethers (PBDEs), and hexabromocyclododecane (HBCD) have the highest consumption figures in the world. TBBPA is produced by bromination of bisphenol-A (Fig. 1). With an estimated global consumption of 210,000 tons in 1999, TBBPA is the most widely used BFR in the world (Alaee *et al.*, 2003). PBDEs are characterized by two brominated phenyl rings, connected by an ether bridge (Fig. 1). As all 10 hydrogen groups of diphenyl ether may be exchanged with bromine during production, theoretically 209 different PBDE congeners can be distinguished. Given the structural similarity between PBDEs and polychlorinated biphenyls (PCBs), PBDEs follow the PCB nomenclature proposed by Ballschmitter and Zell (1980). PBDEs are typically produced in three different commercial mixtures, each with its own average level of bromination, i.e., penta-PBDE, octa-PBDE, and deca-PBDE mixtures. The commercial penta-PBDE mixture Bromkal 70-5DE consists of 80% of only three congeners, i.e., 35% brominated diphenyl ether (BDE) 47, 38% BDE-99, and 7% BDE-100 (Fig. 1) (Sjödin *et al.*, 1998). Octa-PBDE products from different suppliers have different PBDE composition, but in general, hepta-brominated BDE-183 (Fig. 1) is the major

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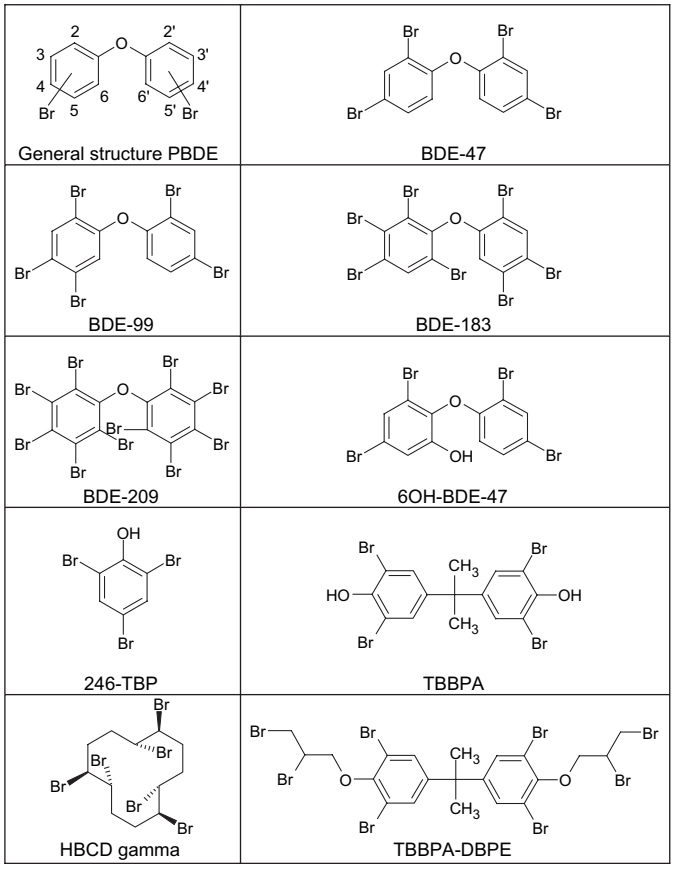

FIG. 1. Molecular structures of BFRs selected for this study. For PBDEs only a general structure and some environmentally relevant representatives are shown.

congener (± 44%; Sjödin, 2000). The deca-PBDE mixture contains > 97% BDE-209 (Fig. 1) (Marsh, 2003). In 1999, global market demands of penta-, octa-, and deca-PBDE mixtures were 8500, 3825, and 54,800 tons, respectively (Alaee *et al.*, 2003). HBCD is the third BFR in production volume with an estimated European market volume of 8950 tons in 1999 (Danish EPA, 1999). HBCD (Fig. 1) is produced by bromination of cyclododecatriene, resulting in three different diastereoisomers (alpha, beta, and gamma). Commercial technical HBCD mixtures typically contain 70–90% of the gamma diastereoisomer (Zegers *et al.*, 2005).

Exposure to BFRs

Several studies have demonstrated that man and wildlife are exposed to BFRs due to their presence in the environment and their bioaccumulating properties (see reviews by Darnerud *et al.*, 2001; Law *et al.*, 2003; Sjödin *et al.*, 2003; Vos *et al.*, 2003). In short, PBDEs, HBCD, and, to a lesser extent, TBBPA are persistent BFRs with bioaccumulating potencies in the aquatic food chain (e.g., Lindberg *et al.*, 2004; Morris *et al.*, 2004; Zegers *et al.*, 2005) and in man (Darnerud *et al.*, 2001; Weiss *et al.*, 2004). In general, PBDEs and HBCD are recognized as persistent organic pollutants in the abiotic environment. HBCD and tetra- to hexa-brominated BDE-47, BDE-99, BDE-100, BDE-153, and BDE-154 also dominate BFR composition in biota (including man), but BDE-209 and TBBPA are hardly found in biota, possibly due to low uptake rate or rapid excretion after biotransformation (Boon *et al.*, 2002; Law *et al.*, 2003).

ED Potency of BFRs

Over the last few years, increasing evidence has become available that some BFRs may have ED potencies (Darnerud *et al.*, 2001; Legler and Brouwer, 2003; Vos *et al.*, 2003). The structural resemblance between TBBPA and PBDEs to thyroxine (T4), which is the precursor of the active thyroid hormone 3,3′,5-triiodothyronine (T3), may explain the reported interaction of these BFRs with the thyroid axis. *In vitro* studies demonstrated the T3-like activity of TBBPA and HBCD (Kitamura *et al.*, 2002, 2005; Schriks *et al.*, 2006; Yamada-Okabe *et al.*, 2005). For TBBPA and hydroxylated PBDEs, Meerts *et al.* (2000) found *in vitro* competition with T4 for binding to human transthyretin (TTR), the T4-transporting protein in plasma. Decreased plasma T4 levels found in rodents exposed to PBDEs (Zhou *et al.*, 2001, 2002) may be attributed to reduced TTR-mediated transport. Alternative explanations are an induced T4 turnover by induced levels of hepatic uridine phosphoglucuronyl transferase, whereas effects on the thyroid gland or thyroid-stimulating hormone levels are less likely (Zhou *et al.*, 2001, 2002). Plasma T4 decreases may be involved in the developmental neurotoxic and behavioral effects described for rodents exposed to BFRs (PBDEs and HBCD) and other polyhalogenated aromatic hydrocarbons (Branchi *et al.*, 2003; Eriksson *et al.*, 2002; Porterfield, 2000; Viberg *et al.*, 2003).

In addition, (anti-)estrogenic properties have been demonstrated *in vitro* for BFRs. Meerts *et al.* (2001) found that the estrogen receptor (ER) was activated by lower-brominated PBDEs and inactivated by higher-brominated (hydroxylated) PBDEs. Kester *et al.* (2002) proposed an alternative (non–ER-regulated) estrogenic mechanism by the inhibition of estradiol sulfotransferase enzymes, leading to a decreased sulfation of estradiol (E2) and an associated increased bioavailability of endogenous estrogens. Estrogenic effects of BFRs have hardly been studied *in vivo*. For TBBPA, estrogenic effects were observed in the mouse uterotropic assay (Kitamura *et al.*, 2005), whereas no effects were found on quail and chicken embryo development (Berg *et al.*, 2001).

Recently, Stoker *et al.* (2005) demonstrated *in vivo* antiandrogenic properties of PBDEs. In a pubertal exposure study with male Wistar rats exposed to the commercial penta-PBDE mixture DE-71, the authors found delayed puberty and a corresponding suppression of ventral prostate and seminal vesicle growth. DE-71 was also found to be antiandrogenic in an immature rat Hershberger assay. Additional *in vitro* studies

indicated that the DE-71 constituents BDE-47, BDE-99, BDE-100, and BDE-154 inhibited binding of the synthetic androgen R1881 to cytosolic androgen receptor (AR) from the ventral prostate. For BDE-100, this inhibition was demonstrated to be purely competitive. Of the four AR-binding PBDE congeners, BDE-47 and BDE-100 also inhibited dihydrotestosterone-induced, human AR-regulated transcriptional activation in a luciferase reporter gene assay (Stoker *et al.*, 2005).

Finally, some PBDE congeners can activate or inactivate the arylhydrocarbon receptor (AhR, or dioxin receptor [DR]; Behnisch *et al.*, 2003; Meerts *et al.*, 1998), which is directly involved in the expression of cytochrome P450-1A. *In vivo*, increased hepatic ethoxyresorufin O-deethylase (EROD) activity has been demonstrated in rats exposed to PBDEs (e.g., Zhou *et al.*, 2001, 2002). With respect to endocrine disruption, induced or inhibited DR-regulated enzyme activities may directly or indirectly affect critical pathways in vitamin A metabolism (Zile, 1992) or steroidogenesis (Fukazawa *et al.*, 2004).

Aim of This Study

Despite indications for bioaccumulating and the ED properties of BFRs, a human and ecological risk assessment for these compounds has been hampered by the lack of a systematic identification and toxicological characterization of the most potent and environmentally relevant BFRs (Vos *et al.*, 2003). The goal of the current study was to perform a systematic *in vitro* screening of the potential ED potencies of BFRs, as part of a hazard identification approach within the EU-funded research program FIRE (RIVM, 2003). For the *in vitro* screening, a test set of 27 individual BFRs were selected, consisting of TBBPA, 19 PBDEs, HBCD (technical mixture [TM] and the alpha, beta, and gamma diastereoisomers), 2,4,6-tribromophenol (246-TBP), and *ortho*-hydroxylated brominated diphenyl ether 47 (6OH-BDE-47). The test set further included tetrabromobisphenol-A–bis(2,3)dibromopropyl ether (TBBPA-DBPE), which is used as a flame-retarding additive for plastics. The subset of PBDEs contained environmentally relevant and also less environmentally relevant congeners. The latter were selected to cover a broad variation in chemical characteristics in the test set, to allow the establishment of quantitative structure-activity relationships (QSARs) that will be described elsewhere (Harju *et al.*, in press). The test set was completed by commercial penta- and octa-PBDE mixtures. All compounds were tested for their potency to interfere via thyroidal, estrogenic, and AhR-mediated pathways. In addition, the potency to interfere with androgenic and progestagenic pathways was tested for the first time for BFRs. Altogether, all compounds were tested in seven different bioassays for 12 endocrine-related modes of action (Table 1). Results from the *in vitro* screening were used for (1) identification of relevant endocrine-related modes of action that may be affected by BFRs in man and wildlife and (2) classification of BFRs with similar ED profiles.

MATERIALS AND METHODS

Brominated flame retardants. BDE-28 [bromine substitution pattern 2,4,4′], BDE-47 [2,2′,4,4′], BDE-49 [2,2′,4,5′], BDE-99 [2,2′,4,4′,5], BDE-100 [2,2′,4,4′,6], BDE-153 [2,2′,4,4′,5,5′], BDE-155 [2,2′,4,4′,6,6′], BDE-169 [3,3′,4,4′,5,5′], BDE-181 [2,2′,3,4,4′,5,6], BDE-183 [2,2′,3,4,4′,5′,6], BDE-190 [2,3,3′,4,4′,5,6], BDE-206 [2,2′3,3′,4,4′,5,5′,6], and BDE-209 [2,2′3,3′,4,4′,5,5′,6,6′] and 6OH-BDE-47 (> 99%; cleaned up on charcoal column to remove possible impurities of brominated dibenzofurans and dioxins) were obtained from Stockholm University through Dr Göran Marsh and Dr Åke Bergman. BDE-19 [2,2′,6], BDE-38 [3,4,5], BDE-39 [3,4′,5], BDE-79 [3,3′,4,5′], BDE-127 [3.3′4,5,5′], and BDE-185 [2,2′,3,4,5,5′,6] (> 98%) were bought from AccuStandard (New Haven CT). Penta-PBDE mixture DE-71 and octa-PBDE mixture Octa LM were obtained from Great Lakes

TABLE 1
Overview of the Seven *In Vitro* Bioassays Used to Determine the Possible ED Potency of BFRs on 12 Different Endpoints Ordered per Endocrine Pathway

Endocrine pathway	Bioassay	Reference material	Endpoint
AhR mediated	DR-CALUX	TCDD	Dioxin-like activity through DR
		2,2′3,3′,4,4′-Hexachlorobiphenyl (PCB-128)	Anti–dioxin-like activity through DR in the presence of TCDD
Androgenic	AR-CALUX	DHT	Androgenic activity through AR
		Flutamide	Antiandrogenic activity through AR in the presence of DHT
Progestagenic	PR-CALUX	MPA	Progestagenic activity through PR
		RU-486	Antiprogestagenic activity through PR in the presence of MPA
Estrogenic	ER-CALUX	17β-E2	Estrogenic activity through ER
		ICI 182,780	Antiestrogenic activity through ER in the presence of E2
	E2SULT	PCP	Inhibited sulfation of E2
Thyroidal	TTR binding	T4	Displacement of thyroid hormone precursor (T4) from its plasma transport protein TTR
	T-Screen	T3	Thyroid hormone–mimicking (T3 like) activity
		Amiodarone	Anti–T3-like activity in the presence of T3

Note. DHT: Dihydrotestosterone; MPA: medroxyprogesterone acetate.

Chemical Corporation (West-Lafayette IL) and Dead Sea Bromine Group (Beer-Sheva, Israel), respectively, and were cleaned up on charcoal column before testing. Based on the PBDE composition of the mixtures (Sjödin, 2000), average molecular weights of 471.9 and 758.1 were estimated for penta- and octa-PBDE mixtures, respectively. TBBPA (> 97%) and 246-TBP (> 98%) were obtained from Aldrich (Milwaukee WI), HBCD (TM containing about 90% gamma diastereoisomer) from Dead Sea Bromine Group, HBCD diastereoisomers (> 97%) from Cambridge Isotope Laboratories (Andover MA), and TBBPA-DBPE (TM) from Broomchemie BV (Terneuzen, Netherlands).

For all compounds, stock solutions were prepared in dimethylsulfoxide (DMSO) by (1) dissolving the compounds in tetrahydrofurane (THF; Sigma-Aldrich [St. Louis MO]; 99.5%), (2) transferring the solutions to DMSO (Acros [Geel, Belgium]; 99.9%), and (3) evaporating the THF under a gentle stream of nitrogen. Final BFR concentrations in the DMSO stocks were 2.5mM, except for BDE-209 for which a stock solution of 1.5mM was prepared due to solubility problems at higher concentrations.

CALUX bioassays. For all test compounds, the potency to interact as an agonist or antagonist with the AhR, the ER, the AR, and the progesterone receptor (PR) was determined in the DR-, ER-, AR-, and PR-CALUX bioassays, respectively (Legler *et al.*, 1999; Murk *et al.*, 1996; Schreurs *et al.*, 2005). All four CALUX bioassays (chemically activated luciferase gene expression; BioDetection Systems BV [BDS], Amsterdam, The Netherlands) make use of reporter cell lines carrying a luciferase gene under the transcriptional control of response elements for activated receptors. The four CALUX bioassays were performed according to protocols from BDS. Methodological differences among the four CALUX bioassays are indicated in Table 2. In short, cells were plated in 96-well plates with the medium supplemented with fetal calf serum (FCS; Table 2). After a given period of time (see Table 2), the medium was refreshed with exposure medium containing the BFR to be tested (maximum concentration 10 or 12.5μM), in the absence or in the presence of a reference agonist. After 24 h of incubation, the medium was removed; cells were lysed in 30 μl of Triton-lysis buffer and measured for luciferase activity using a luminometer.

Estradiol sulfotransferase assay. The estradiol sulfotransferase (E2SULT)–inhibiting potency of BFRs was determined according to Kester *et al.* (2000) with minor modifications. In short, the effect of BFRs on the formation of E2 sulfate was determined after incubation of tritium-labeled estradiol (^{3}H-E2), sulfotransferase, and the cofactor 3′-phosphoadenosine-5′-phosphosulfate (PAPS). Recombinant human sulfotransferase 1E1 (SULT1E1) expressed in a V79-1E1 Chinese hamster cell line (Czich *et al.*, 1994) was kindly provided by Prof. Dr H. Glatt (German Institute of Human Nutrition). SULT1E1 was incubated (37°C; 30 min; 2 μg cytosolic protein/ ml) with 1nM ^{3}H-E2 (2,4,6,7,16,17-^{3}H-estradiol; Amersham Biosciences [Buckinghamshire, UK]) and 50μM PAPS (Sigma-Aldrich) in 0.2 ml of 0.1M sodium phosphate (pH 7.2), 2mM EDTA, and 1mM dithiothreitol in the presence or absence (control) of the test compound (maximum concentration 10μM) or pentachlorophenol (PCP), used as a reference material (Kester *et al.*, 2000). Maximum concentration of the test compounds during incubation was 10μM, and all incubations contained 1% DMSO. Blank incubations received no PAPS. The reaction was stopped by adding 800 μl of ice-cold water. Unreacted ^{3}H-E2 was removed by extraction of the incubation mixture with 2 ml of dichloromethane. The amount of water-soluble ^{3}H-E2 sulfate was quantified by counting 0.5 ml of the water phase in a beta counter (LKB Wallack 1219 Rackbeta).

TTR-binding assay. The capacity of BFRs to compete with T4 for binding to TTR was investigated according to Lans *et al.* (1993) with modifications. Human TTR (prealbumin from human plasma, Sigma-Aldrich; 30nM) was incubated overnight at 4°C in a final volume of 200 μl 0.1M Tris-HCl buffer (pH 8.0; 0.1M NaCl, 0.1mM EDTA) with a mixture of ^{125}I-labeled (L-3′-5′-^{125}I-thyroxine, Amersham Biosciences) and -unlabeled (Sigma-Aldrich) T4 (55nM; 100,000 cpm) and test compounds (maximum concentration 62.5μM) or cold T4 (reference material) as competitors. All treatments and carrier controls contained 2.5% DMSO. When binding equilibrium was reached, TTR-bound and free ^{125}I-T4 were separated on 1-ml Biogel P-6PG columns and spin-force eluted with 200 μl Tris-HCl buffer (1 min, 100g, 4°C). The TTR-bound ^{125}I-T4–containing eluate was counted for radioactivity on a gamma counter (LKB Wallack 1282 Compu-gamma CS) and corrected for the initial amount of ^{125}I-T4 added to the incubation mixture that had been counted before incubation started.

T-Screen. BFR potency to act as a thyroid hormone receptor (TR) agonist or antagonist was tested in the T-Screen (Gutleb *et al.*, 2005), a functional assay

TABLE 2
Differences in Experimental Characteristics of the CALUX Assays

Experimental characteristic	DR-CALUX	AR-CALUX	PR-CALUX	ER-CALUX
Cell type	H4IIE (rat hepatoma)	U-2 OS (human osteoblast)	U-2 OS (human osteoblast)	T47D (human breast cancer)
Initial cell density (cells per well)	Not determined[a]	6000	4000	7500
Medium (GIBCO)	α-MEM[b] with phenol red	Phenol red–free DF medium	Phenol red–free DF medium	Phenol red–free DF medium 1.26 g/l $NaHCO_3$ 1× nonessential amino acids
FCS (GIBCO) (% in medium)	10%	5% DCC[b]	5% DCC[b]	5% DCC[b]
Time between plating and exposing of the cells (h)	24	48	48	48
Final concentration (pM) of reference agonist added (1–2 × EC_{50}) to determine antagonistic potency[c]	15	164	98	6
Final DMSO concentration (% vol/vol)	0.4	0.2	0.2	0.5
Maximum BFR concentration (μM)	10	10	10	12.5
Final test volume (μl)	200	200	200	100

[a]Twenty-four hours after plating, the cells were confluent at the start of the exposure period.
[b]MEM: minimum essential medium; DCC: dextran-coated charcoal stripped.
[c]See Table 3.

based on the T3-dependent cell proliferation of a rat pituitary tumor cell line (GH3) when plated at low density in serum-free medium. GH3 cells were cultured (humid atmosphere, 37°C, 5% CO_2) in Dulbecco's modified Eagle's medium/Ham's F12 (DF medium; GIBCO [Breda, Netherlands]) supplemented with 10% FCS (GIBCO) and 15mM HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid). Prior to BFR exposure, cells were incubated for 48 h in serum-free DF medium to deplete cells of thyroid hormones. On the day of exposure, cells were plated on a transparent 96-microtiter plate (Greiner [Alphen aan de Rijn, Netherlands]; 2500 cells per well). GH3 cells were exposed in triplicate to a full dose-response range (0.001–1000nM) of reference material T3 or test chemicals (in the absence or presence of 0.25nM T3 [EC_{50}]) in fresh DF medium. All treatments and carrier controls contained 0.5% DMSO. After 96 h of exposure, cell proliferation was determined by measuring the total metabolic activity of the cells by adding 8 µl of resazurin (400µM in PBS; Sigma-Aldrich) to each well. After 4 h of incubation (37°C; 5% CO_2), formation of the fluorescent metabolite resorufin was determined in a fluorometer (Milli-pore Cytofluor 2350; $\lambda_{ex} = 530$ nm; $\lambda_{em} = 590$ nm) (O'Brien *et al.*, 2000).

Cytotoxicity. To ensure that reduced activities in the various bioassays were caused by true antagonistic responses and not by cytotoxicity, viability was measured in DR-CALUX and ER-CALUX cells exposed to 10 or 12.5µM BFR, which was the maximum concentration tested in the CALUX assays (Table 2). As in the T-Screen, cell viability was measured as the metabolic capacity to reduce blue resazurin into pink resorufin (O'Brien *et al.*, 2000). CALUX cells were plated as in the original CALUX assay. CALUX cells were exposed for 24 h to exposure medium (cell-specific medium containing BFR and 40µM resazurin), and metabolic capacity was determined spectrophotometrically (SpectraMAX 340 PC) as a decrease of the blue-colored resazurin ($\lambda = 600$ nm) in the assay medium.

Data analysis. For all bioassays, dose-response curves were determined for those compounds showing effects > 20% of control level in earlier range-finding experiments. EC_{50} or IC_{50} values were calculated for those compounds for which a full dose-response curve could be constructed. For compounds yielding responses between 10 and 50% of control level within the range of test concentrations, EC_{50} or IC_{50} values were either extrapolated from the dose-response curve (in case the extrapolated value was reasonably close to the highest test concentration) or set at a minimum value (see Tables 4–8). In most bioassays, dose-response curves reached maximum responses (sometimes after extrapolation) comparable to the reference compound. For only a few DR-agonistic and E2SULT-inhibiting BFRs, maximum responses were lower than for reference compounds. In these cases (indicated by footnotes in Tables 4 and 7, respectively), EC_{50} or IC_{50} values were calculated as the concentration at the point of inflection in the compound-specific dose-response curve and not as the concentration corresponding with 50% of the maximum response induced by the reference material. *In vitro*, ED potencies of all BFRs were classified according to the criteria listed in Table 3. Classification was based on EC_{50} or IC_{50} values, except for T-Screen results, which did not allow EC_{50} estimation due to cytotoxicity. For each BFR, a compound-specific toxicity profile was constructed including a set of 12 classified ED activities determined *in vitro*.

Toxicity profiles of all 27 BFRs were analyzed for common features using two different pattern recognition methods: hierarchical cluster analysis and principal component analysis (PCA). In both methods, the BFR-specific toxicity profile is considered as a set of coordinates that define the position of the BFR in a 12-dimensional space. Two different pattern recognition methods were chosen to improve interpretation and to verify results from one method with those from the other method.

Hierarchical cluster analysis (e.g., Aldender and Blashfield, 1984) is a pattern recognition method that is solely based on the calculated distances between the 27 BFRs within this 12-dimensional space. In the first stage, the two BFRs with lowest distance are grouped together into a cluster. Next, an average location for this cluster is calculated in the 12-dimensional space. In the second stage, distances between the remaining 25 BFRs and the new cluster are calculated and cases with lowest distance merged again. This can be a third and a fourth BFR that merge into a second cluster, but also a third BFR that is added to the first cluster. This procedure is repeated, adding BFRs to existing clusters, creating new clusters, or combining clusters until only one cluster is left. Results from a hierarchical cluster analysis may be presented in a so-called dendrogram (see Fig. 3), a diagram that illustrates the fusions made at successive stages of analysis and indicates the relative distance between the different BFRs and clusters in the 12-dimensional space. Hierarchical cluster analysis was performed in SPSS (version 10.0 for Windows) based on squared Euclidean distances between class values using the between-group linkage method.

In PCA (e.g., Jackson, 1991), the 27 BFRs located in the 12-dimensional space are projected down to a lower-dimensional hyperspace. In Figure 4, a score plot is shown where two latent explanatory variables (principal components or PCs) are used as x-axis and y-axis. These two orthogonal vectors are calculated in such a way that PC1 explains the largest part of the total variance, PC2 the largest part of the remaining variance, etc. The score plot yields an overview of the major patterns in the data set, and BFRs found closely together have a similar toxicity profile. The distance of a particular BFR to the origin indicates its deviation from the toxicity profile of the "average BFR." Similarly, the significance of the original 12 bioassay responses can be illustrated in a so-called loading plot, where highly significant ED potencies have a larger distance to the origin than less-significant potencies. By superimposing the loading plot on the score plot (as done in Fig. 4), correlations between BFRs and bioassay responses can be visualized: BFRs and bioassay responses located closely together are positively correlated, whereas BFRs and bioassay responses with opposite positions are negatively correlated. The PCA calculations were performed in Simca-P+ 10.0 (Umetrics Inc, Umeå, Sweden), using a cutoff in eigenvalue of 2 to define the number of significant components.

RESULTS

CALUX Bioassays

Of the nine DR-agonistic PBDEs, only four (BDE-38, BDE-153, BDE-190 and 6OH-BDE-47) were able to induce a full

TABLE 3
Criteria for Classification of BFRs Based on *In Vitro* Toxicity Results

Class	Potency	Criteria (all bioassays except T-Screen potentiation)	Criteria T-Screen potentiation[a]
1	No potency	Response < 20% of control at 10µM	Additional response < 10%
2	Low potency	(EC_{50} or IC_{50}) > 10µM and response > 20% of control at 10µM	10% < additional response < 20%
3	Moderate potency	1µM < (EC_{50} or IC_{50}) < 10µM	20% < additional response < 30%
4	High potency	0.1µM < (EC_{50} or IC_{50}) < 1.0µM	30% < addition response < 40%
5	Very high potency	0.01µM < (EC_{50} or IC_{50}) < 0.1µM	40% < additional response

[a]Additional response as the percentage of maximum T3 response at the highest noncytotoxic concentration tested (0.5 or 1µM).

dose-response curve up to the maximum induction found for the most potent DR ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Table 4). The remaining five DR-agonistic PBDEs (BDE-49, BDE-79, BDE-99, BDE-181, and BDE-183) reached maximum induction levels between 52 and 63% of the TCDD induction maximum and were therefore considered as partial agonists (Fig. 2). Based on EC_{50} values, BDE-38 was the most potent compound, with a DR-agonistic potency of about 56,000 times less than that of TCDD (Table 4). DR-antagonistic activity was found for lower-brominated PBDEs (up to hexa-brominated BDE-169) and to some extent for HBCD (Table 4). BDE-47 was the most potent DR antagonist tested (IC_{50} = 2.7μM), with activities similar to the reference antagonist PCB-128 (Fig. 2; Table 4).

No agonistic AR and PR activity could be demonstrated for any of the BFRs tested. However, for 22 out of the 27 BFRs, antiandrogenic and—to a lesser extent—antiprogestagenic activities were found (Table 5). Only BDE-169, BDE-206, BDE-209, TBBPA, and TBBPA-DBPE did not show any AR- and PR-antagonistic activity. Highest AR antagonism was found for BDE-19 and BDE-100 with IC_{50} values of 0.060 and 0.097μM, respectively (Table 5; Fig. 2). Together with BDE-47 and BDE-49, these BFRs were more potent than the antiandrogenic drug flutamide (IC_{50} = 1.3μM) that was used as a reference AR-antagonistic compound (Sonneveld *et al.*, 2005). PR-antagonistic activity was also highest for BDE-19 (IC_{50} = 0.8μM). In addition, IC_{50} values of ≤ 5μM were found for HBCD (TM and gamma diastereoisomer), BDE-100, BDE-155, BDE-181, BDE-183, BDE-185, BDE-190, and 6OH-BDE-47 (Table 5). All PR-antagonistic BFRs were more than three orders of magnitude less potent than the pregnancy-terminating and breast cancer drug RU-486 that was used as an

TABLE 4
AhR (DR)-Agonistic and -Antagonistic Responses of the 27 BFRs and Positive Controls TCDD and PCB-128 in the DR-CALUX Assay[a]

	DR agonistic		DR antagonistic	
Compound	EC_{50} (μM) ± SD	TCDD EF ± SD	IC_{50} (μM) ± SD	PCB-128 EF SD
BDE-19	—	—	—	—
BDE-28	—	—	4.5 ± 0.4	0.66 ± 0.05
BDE-38	0.5 ± 0.0	$18 \pm 3 \times 10^{-6}$	—	—
BDE-39	—	—	13 ± 1	0.22 ± 0.02
BDE-47	—	—	2.7 ± 0.7	1.1 ± 0.3
BDE-49	> 15[b]	$< 0.6 \times 10^{-6}$	> 15	< 0.20
BDE-79	1.4 ± 0.3[b]	$5.8 \pm 0.2 \times 10^{-6}$	—	—
BDE-99	> 15[b]	$< 0.6 \times 10^{-6}$	13 ± 0	0.22 ± 0.01
BDE-100	—		> 15	< 0.20
BDE-127	—	—	9.7 ± 0.9	0.31 ± 0.03
BDE-153	0.6 ± 0.2	$15 \pm 2 \times 10^{-6}$	—	—
BDE-155	—	—	—	—
BDE-169	—	—	6.2 ± 4.4	0.64 ± 0.46
BDE-181	4.5[b] ± 2.7	$2.4 \pm 1.9 \times 10^{-6}$	—	—
BDE-183	2.0[b] ± 0.1	$4.4 \pm 1.2 \times 10^{-6}$	—	—
BDE-185	—	—	—	—
BDE-190	1.2 ± 0.3	$7.1 \pm 0.1 \times 10^{-6}$	—	—
BDE-206	—	—	—	—
BDE-209	—	—	—	—
TBBPA	—	—	—	—
246-TBP	—	—	—	—
6OH-BDE-47	1.3 ± 0.4	$6.9 \pm 0.8 \times 10^{-6}$	—	—
HBCD TM	—	—	> 15	< 0.20
HBCD alpha	—	—	7.4 ± 1.8	0.42 ± 0.10
HBCD beta	—	—	> 15	< 0.20
HBCD gamma	—	—	11 ± 2	0.29 ± 0.04
TBBPA-DBPE	—	—	—	—
Penta-mix	—	—	4.5 ± 1.2	0.68 ± 0.17
Octa-mix	—	—	2.8 ± 0.1	1.1 ± 0.0
TCDD	$8.3 \pm 1.5 \times 10^{-6}$	1.0 ± 0.2	ND	ND
PCB-128	ND	ND	3.0	1

[a] $n \geq 2$; —: no response; ND: not determined.
[b] Partial agonists reaching maximum induction levels 52–63% of the maximum TCDD induction.

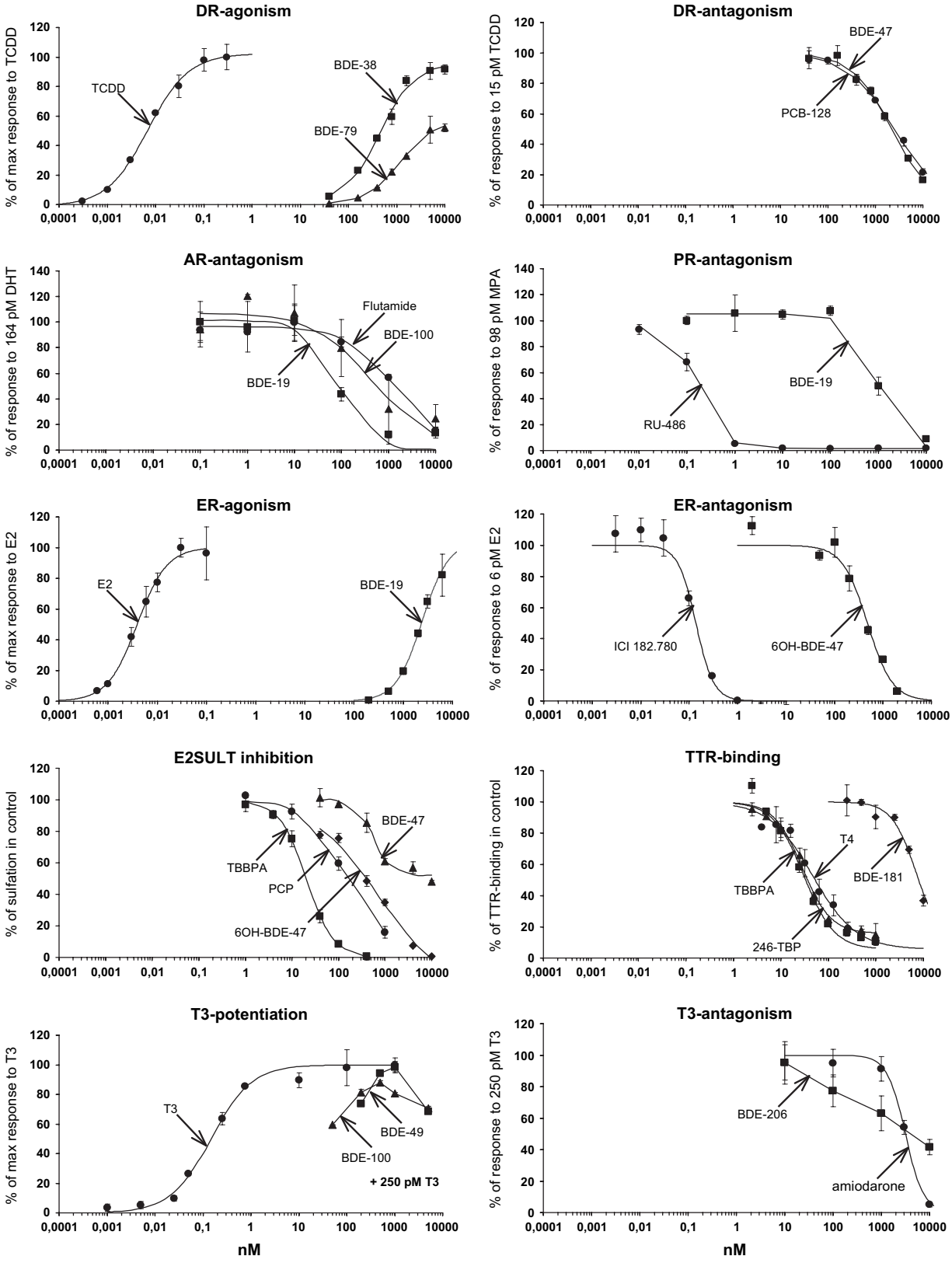

FIG. 2. Typical examples of dose-response relationships determined for BFRs in the *in vitro* bioassays: agonistic and antagonistic responses towards the DR, AR, PR, and ER; inhibition of E2SULT; competition with T4 for TTR binding; potentiation and antagonism of T3-mediated effects. For each mode of action, results are presented for a reference compound (●) and one or more exemplary BFRs (■, ▲, ♦).

TABLE 5
Antiandrogenic and Antiprogestagenic Potency of the 27 BFRs and Positive Controls Flutamide and RU-486 in the AR- and PR-CALUX Assays[a]

	AR antagonistic		PR antagonistic	
Compound	IC_{50} (μM) ± SD	Flutamide EF	IC_{50} (μM) ± SD	RU-486 EF
BDE-19	0.060 ± 0.002	21.6	0.8 ± 0.4	2.6×10^{-4}
BDE-28	3.1 ± 0.7	0.41	> 15	$< 0.2 \times 10^{-4}$
BDE-38	1.9 ± 0.6	0.69	> 15	$< 0.2 \times 10^{-4}$
BDE-39	3.5 ± 0.3	0.37	> 15	$< 0.2 \times 10^{-4}$
BDE-47	1.0 ± 0.2	1.29	> 15	$< 0.2 \times 10^{-4}$
BDE-49	0.67 ± 0.5	1.95	6.1[b]	0.3×10^{-4}
BDE-79	2.0 ± 0.6	0.66	> 15	$< 0.2 \times 10^{-4}$
BDE-99	7.8 ± 3.3	0.17	> 15	$< 0.2 \times 10^{-4}$
BDE-100	0.10 ± 0.01	13.4	3.4 ± 2.4	0.6×10^{-4}
BDE-127	5.1 ± 4.5	0.26	> 15	$< 0.2 \times 10^{-4}$
BDE-153	13.2[b]	0.1	5.7 ± 1.8	0.4×10^{-4}
BDE-155	2.0[b]	0.64	3.8 ± 2.4	0.5×10^{-4}
BDE-169	—		—	
BDE-181	3.0[b]	0.43	3.6 ± 1.4	0.6×10^{-4}
BDE-183	> 15	< 0.1	2.6[b]	0.8×10^{-4}
BDE-185	> 15	< 0.1	3.6 ± 0.2	0.6×10^{-4}
BDE-190	8.8[b]	0.15	4.8[b]	0.4×10^{-4}
BDE-206	—		—	
BDE-209	—		—	
TBBPA	—		—	
246-TBP	> 15[b,c]	< 0.1	> 15	$< 0.2 \times 10^{-4}$
6OH-BDE-47	2.8 ± 0.4[c]	0.46	5.0 ± 0.9[c]	0.4×10^{-4}
HBCD TM	> 15	< 0.1	1.6 ± 0.0	1.3×10^{-4}
HBCD alpha	3.4[b]	0.38	8.5 ± 1.5	0.2×10^{-4}
HBCD beta	11.6 ± 3.7[c]		> 15	$< 0.2 \times 10^{-4}$
HBCD gamma	3.7 ± 1.0	0.35	1.4 ± 0.0	1.5×10^{-4}
TBBPA-DBPE	—	—	—	
Penta-mix	2.0[b]	0.65	10[b]	0.2×10^{-4}
Octa-mix	> 15[b]	< 0.1	> 15[b]	$< 0.2 \times 10^{-4}$
Flutamide	1.3	1.0	ND	ND
RU-486	ND	ND	0.0002	1.0

[a] $n \geq 2$; —: no response; ND: not determined.
[b] $n = 1$.
[c] Cytotoxicity was not observed, but cannot be excluded.

antiprogestagenic reference compound (Mahajan and London, 1997).

ER-agonistic (i.e., estrogenic) responses were only found for low-brominated diphenyl ethers (up to hexa-brominated BDE-155), with EC_{50} values > 2μM (Table 6). Estrogenic potency of BFRs was six orders of magnitude lower than for the natural ligand E2, with highest potency for BDE-19 (EC_{50} = 2.4μM; Fig. 2) for which a molar E2 equivalency factor (E2 EF) of 1.26×10^{-6} was found (Table 6). ER-antagonistic (i.e., anti-estrogenic) responses were found for tetra-brominated BDE-79, all hepta-brominated PBDEs in the test set (i.e., BDE-181, BDE-183, BDE-185, and BDE-190), HBCD, and the phenolic compounds 6OH-BDE-47 and 246-TBP (but not TBBPA). The most potent test compound was 6OH-BDE-47 (IC_{50} = 0.5μM; Fig. 2), which was 3000 times less potent than the antiestrogenic drug ICI 182.780 (Table 6) that was used a reference compound (Legler *et al.*, 1999).

E2SULT Assay

TBBPA was the most potent inhibitor of E2 sulfation (IC_{50} = 0.016μM), being almost 13 times more potent than the well-known inhibitor PCP (Table 7; Fig. 2). 246-TBP and to a lesser extent 6OH-BDE-47 were also potent E2SULT inhibitors, indicating that the E2SULT-inhibiting potency is supported by the presence of a hydroxylated aromatic group. Although full dose-response curves were obtained within the range of test concentrations, maximum E2SULT-inhibiting potencies of only 60, 50, and 40% were found for the lower-brominated BDE-19, BDE-47, and BDE-49, respectively (see BDE-47 in

TABLE 6
Estrogenic and Antiestrogenic Responses of the 27 BFRs and Positive Controls E2 and ICI 182.780 in the ER-CALUX Assay[a]

	ER agonistic		ER antagonistic	
Compound	EC_{50} (μM) ± SD	E2 EF ± SD	IC_{50} (μM) ± SD±	ICI 182.780 EF ± SD
BDE-19	2.4 ± 0.1	$1.3 \pm 0.3 \times 10^{-6}$	—	—
BDE-28	> 15	$< 0.27 \times 10^{-6}$	—	—
BDE-38	6.2 ± 1.0	$0.48 \pm 0.07 \times 10^{-6}$	—	—
BDE-39	—	—	—	—
BDE-47	12 ± 4	$0.35 \pm 0.17 \times 10^{-6}$	—	—
BDE-49	9.2 ± 0.2	$0.47 \pm 0.13 \times 10^{-6}$	—	—
BDE-79	> 15	$< 0.27 \times 10^{-6}$	> 15	$< 9.2 \times 10^{-6}$
BDE-99	—	—	—	—
BDE-100	7.0 ± 2.3	$0.55 \pm 0.22 \times 10^{-6}$	—	—
BDE-127	—	—	—	—
BDE-153	—	—	—	—
BDE-155	8.1 ± 0.9	$0.52 \pm 0.08 \times 10^{-6}$	—	—
BDE-169	—	—	—	—
BDE-181	—	—	4.7 ± 2.1	$32 \pm 14 \times 10^{-6}$
BDE-183	—	—	6.4 ± 2.9	$24 \pm 11 \times 10^{-6}$
BDE-185	—	—	13 ± 7	$12 \pm 6 \times 10^{-6}$
BDE-190	—	—	1.9 ± 1.0	$83 \pm 43 \times 10^{-6}$
BDE-206	—	—	—	—
BDE-209	—	—	—	—
TBBPA	—	—	—	—
246-TBP	—	—	8.3 ± 1.2[b]	$17 \pm 2 \times 10^{-6}$
6OH-BDE-47	—	—	0.45 ± 0.03[b]	$304 \pm 22 \times 10^{-6}$
HBCD TM	—	—	> 15	—
HBCD alpha	—	—	—	—
HBCD beta	—	—	11 ± 3	$13 \pm 3 \times 10^{-6}$
HBCD gamma	—	—	4.9 ± 0.1	$29 \pm 1 \times 10^{-6}$
TBBPA-DBPE	—	—	—	—
Penta-mix	—	—	—	—
Octa-mix	—	—	> 15	$< 9.2 \times 10^{-6}$
E2	$4.0 \pm 1.0 \times 10^{-6}$	1.0 ± 0.3	ND	ND
ICI 182.780	ND	ND	0.14×10^{-3}	1.0

[a] $n \geq 2$; —: no response; ND: not determined.
[b] Cytotoxicity was not observed, but cannot be excluded.

Fig. 2). This probably resulted in an overestimation of their E2SULT-inhibiting potency when BFRs were classified according to their IC_{50} values (see below).

Thyroid-Related Assays

246-TBP and TBBPA were also very potent T4 competitors in the TTR-binding assay (IC_{50} < 0.1μM) with a 10.2 and 1.6 times higher TTR-binding potency than the natural ligand T4 (Fig. 2; Table 7). While 6OH-BDE-47 is the third most potent inhibitor in order and nonhydroxylated PBDEs and HBCD have much lower T4-competing potency, a hydroxylated aromatic group seems to be required for TTR-binding capacity.

In the absence of T3, the T-Screen–agonistic activity was found for HBCD alpha at 1μM (up to 21% of the maximum T3-induced proliferation; Fig. 2) and to a lesser extent for BDE-127 and BDE-185 (Table 8). When tested in the presence of 0.25nM T3, however, 13 BFRs were able to increase the T3 response from 50 to 94% of the maximum T3-induced response. This potentiating effect was most pronounced for BDE-49, BDE-100, and HBCD alpha and gamma diastereoisomers (Fig. 2, Table 8). In contrast, BDE-206 caused a significant decrease in T3-induced cell proliferation at concentrations exceeding 0.1μM (Fig. 2).

Cytotoxicity

For all BFRs, microscopic observations revealed visible cytotoxicity in GH3 cells (T-Screen) at concentrations ≥ 1μM, whereas no cytotoxicity was seen in any of the CALUX cells at maximum test concentrations. Since antagonistic responses in cell-based bioassays could be competed out in most cases by excess of the reference agonist (100 × EC_{50}; data not shown), inhibition could be attributed to true antagonism (i.e., competition for the receptor-binding sites) and not to cytotoxicity. Antagonistic responses of 6OH-BDE-47, 246-TBP, and HBCD

TABLE 7
T4-TTR Competing Potency and E2SULT-Inhibiting Potency of the 27 BFRs and Positive Controls T4 and PCP in the TTR and E2SULT Assays[a]

	T4 replacement		E2SULT inhibition	
Compound	IC_{50} (μM)	T4 EF	IC_{50} (μM) ± SD	PCP EF ± SD
BDE-19	—		2.7 ± 0.0[b]	0.07 ± 0.03
BDE-28	—		> 10	< 0.015
BDE-38	> 25	$< 2.2 \times 10^{-3}$	—	
BDE-39	—		—	
BDE-47	> 25	$< 2.2 \times 10^{-3}$	0.8 ± 0.4[b]	0.23 ± 0.03
BDE-49	> 25	$< 2.2 \times 10^{-3}$	1.3[b]	0.18
BDE-79	—		—	
BDE-99	—		—	
BDE-100	—		> 15	< 0.010
BDE-127	22		> 10	< 0.015
BDE-153	—		—	
BDE-155	—		> 15	< 0.010
BDE-169	> 25	$< 2.2 \times 10^{-3}$	> 10	< 0.015
BDE-181	7.0	10×10^{-3}	—	
BDE-183	—		> 10	< 0.015
BDE-185	7.4		—	
BDE-190	9.0	8.0×10^{-3}	3.8 ± 1.6	0.052 ± 0.049
BDE-206	—		> 10	
BDE-209	—		> 10	< 0.015
TBBPA	0.031	1.6	0.016 ± 0.007	13 ± 12
246-TBP	0.0048	10	0.27 ± 0.23	0.82 ± 0.41
6OH-BDE-47	0.18	0.26	1.4 ± 1.5	0.18 ± 0.19
HBCD TM	—		—	
HBCD alpha	12	2.7×10^{-3}	—	
HBCD beta	25	2.3×10^{-3}	—	
HBCD gamma	—		—	
TBBPA-DBPE	5.2	8.6×10^{-3}	0.27 ± 0.11	0.87 ± 0.46
Penta-mix	—	—	> 15	< 0.010
Octa-mix	—	—	—	
T4	0.055	1.0	ND	ND
PCP	ND	ND	0.15 ± 0.06	1.0 ± 0.4

[a] $n \geq 2$; —: no response; ND: not determined.
[b] E2SULT inhibitors reaching maximum inhibition levels 42–64% of the maximum PCP inhibition.

beta diastereoisomer in some CALUX assays could not be competed out by excess of agonists (see footnotes in Tables 5 and 6). In these few cases, cytotoxicity may have contributed to the antagonistic response, although microscopic cell damage was not observed. Also, additional cell viability assays with resazurin did not indicate cytotoxicity for these ''suspected'' BFRs in DR- and ER-CALUX cells.

Toxicity Profiling

Cytotoxicity in the T-Screen at BFR-concentrations > 1μM hampered the calculation of full dose-response curves with EC_{50} values for T3 potentiation. To enable the inclusion of T-Screen results in a toxicity-profiling analysis together with those from the other bioassays for which EC_{50} or IC_{50} were available, all bioassay results were classified into dimensionless classes according to predefined criteria (Table 3). As is the case with EC_{50} or IC_{50} values, these classes only reflect relative differences in ED potency of BFRs within one single bioassay, but not among different bioassays.

After classification, compound-specific toxicity profiles were established, which were further arranged into five different clusters of BFRs using hierarchical cluster analysis (Fig. 3). Cluster I consists of BFRs with moderate to very high TTR-binding affinity and high to very high E2SULT-inhibiting potency. This category includes the phenolic compounds 246-TBP and TBBPA and the TBBPA derivative TBBPA-DBPE. Cluster II consists of BDE-19, BDE-49, BDE-100, and BDE-155 with moderate to very high antiandrogenic activity, moderate to high antiprogestagenic and T3-potentiating activities, and moderate estrogenic activity. Cluster III consists of HBCD (TM and the three individual diastereoisomers) and BDE-28 with moderate to high T3-potentiating activity and low to moderate antiandrogenic and antiprogestagenic activity

TABLE 8
T3-Potentiating and T3-Antagonistic Potency of the 27 BFR (1μM) in the T-Screen[a]

	Without T3	With EC_{50} T3 (0.25nM)
Compound	% Effect ± SD	Additional % effect ± SD
BDE-19	—	22[b] ± 1
BDE-28	—	33 ± 3
BDE-38	—	14[c]
BDE-39	—	—
BDE-47	—	—
BDE-49	—	44 ± 3
BDE-79	—	—
BDE-99	—	—
BDE-100	—	38[b] ± 1
BDE-127	14 ± 2	14 ± 1
BDE-153	—	—
BDE-155	—	27 ± 4
BDE-169	—	—
BDE-181	—	—
BDE-183	—	21[b] ± 0
BDE-185	12 ± 2	—[b]
BDE-190	—	—[b]
BDE-206	—	−28[b] ± 2
BDE-209	—	—
TBBPA	—	23[b,c]
246-TBP	—	—
6OH-BDE-47	—[b]	cytotoxic
HBCD TM	—	24 ± 6
HBCD alpha	21 ± 3	38 ± 3
HBCD beta	—	26[c]
HBCD gamma	—	40[c]
TBBPA-DBPE	—	—

[a] $n \geq 2$; —: no response.
[b] Determined at 0.5μM due to cytotoxicity at 1μM.
[c] $n = 1$.

in combination with low to moderate DR-antagonistic activity. Cluster IV consists of tri- to hepta-brominated PBDEs and 6OH-BDE-47 with moderate to high DR-agonistic activity, in combination with low to moderate antiandrogenic and antiprogestagenic activities. This cluster includes a subset (IV.2) of 6OH-BDE-47, BDE-183, and BDE-190 with a very similar profile of moderate to high ED potencies for 6 of the 12 endpoints tested (AR, PR, and ERantagonism, DR agonism, E2SULT inhibition, and TTR-binding affinity). Cluster V seems to consist of ''rest'' BFRs and can be divided into BFRs without (V.1) and with (V.2) AR- and PR-antagonistic activities. BDE-47 seems to be an outlier for this cluster of BFRs, as can be seen from its relatively long distance from the other BFRs in cluster V.

PCA was performed on the classified toxicity profiles based on the 10 ED endpoints for which a response was found for any of the 27 BFRs tested (i.e., omitting the negative AR- and PR-agonistic results found for all tested BFRs). Results from the PCA are shown in Figure 4 as a two-dimensional score plot with superimposed loading plot (see ''Materials and Methods'' section). To allow a direct comparison between results from both pattern recognition methods, BFR markers in the PCA plot have the same color as their corresponding cluster distinguished in the hierarchical cluster analysis in Figure 3 (first column). The first PC, PC1, explained 29% of the variation in the data set. PC1 is clearly associated with differences in TTR-binding and E2SULT-inhibiting potencies (cluster I) on the one hand (left side of Fig. 4) and AR- and PR-antagonistic, ER-agonistic, and T-Screen–potentiating potencies (cluster II) on the other hand (right side of Fig. 4). The second PC, PC2, explained 21% of the variation and seems to be associated with differences in DR-agonistic potencies (cluster IV; top side of Fig. 4) and DR-antagonistic potencies on the other side (clusters III and V; bottom side of Fig. 4). The third PC, PC3, had a low significance (eigenvalue < 2) and explained only 16% of the variation. PC3 is not shown in the two-dimensional biplot of Figure 4, but the marker triangles for BFRs and bioassay endpoints point in the direction by which they were oriented in the third dimension. As can be seen from Figure 4, PC3 roughly distinguishes between BFRs with specific high potencies (clusters I and II) or broad potencies (cluster IV.2) on the one hand (markers pointing up in Fig. 4) and all remaining BFRs on the other hand (markers pointing down). PC3 especially distinguishes among BFRs located in the left side of Figure 4, separating potent TTR-binding and E2SULT-inhibiting BFRs from the inactive BDE-169 and BDE-209 and the T3-antagonistic BDE-206.

DISCUSSION

In Vitro *ED Potency of BFRs*

The *in vitro* screening demonstrated that ED mechanisms of action that were only described for a few BFRs so far, are shared by a wider set of BFRs than ever tested before. For instance, AR-antagonistic potencies recently demonstrated for penta-PBDE mixture DE-71 and BDE-100 (Sonneveld *et al.*, 2005; Stoker *et al.*, 2005) were found for 18 out of the 27 BFRs in the test set (IC_{50} < 15μM; Table 4). In addition, AR-antagonistic responses were among the highest of all bioassay responses determined in the present study, with maximum potencies for BDE-19 and BDE-100 being higher than for flutamide, an antiandrogenic drug used in prostate cancer treatment. Given the presence of BDE-100 in the penta-PBDE mixture, this relatively high AR-antagonistic potency seems to be especially relevant. Using a QSAR model based on the AR-CALUX results of the present study, Harju *et al.* (in press) predicted AR-antagonistic activity (IC_{50} = 0.1–3.1μM) for the nontested penta-PBDE mixture constituents BDE-17, BDE-66, BDE-85, and BDE-154. In general, the QSAR model predicted high AR-antagonistic potency for low-brominated PBDEs with *ortho* or *ortho-para* substitution. For TBBPA, the present study

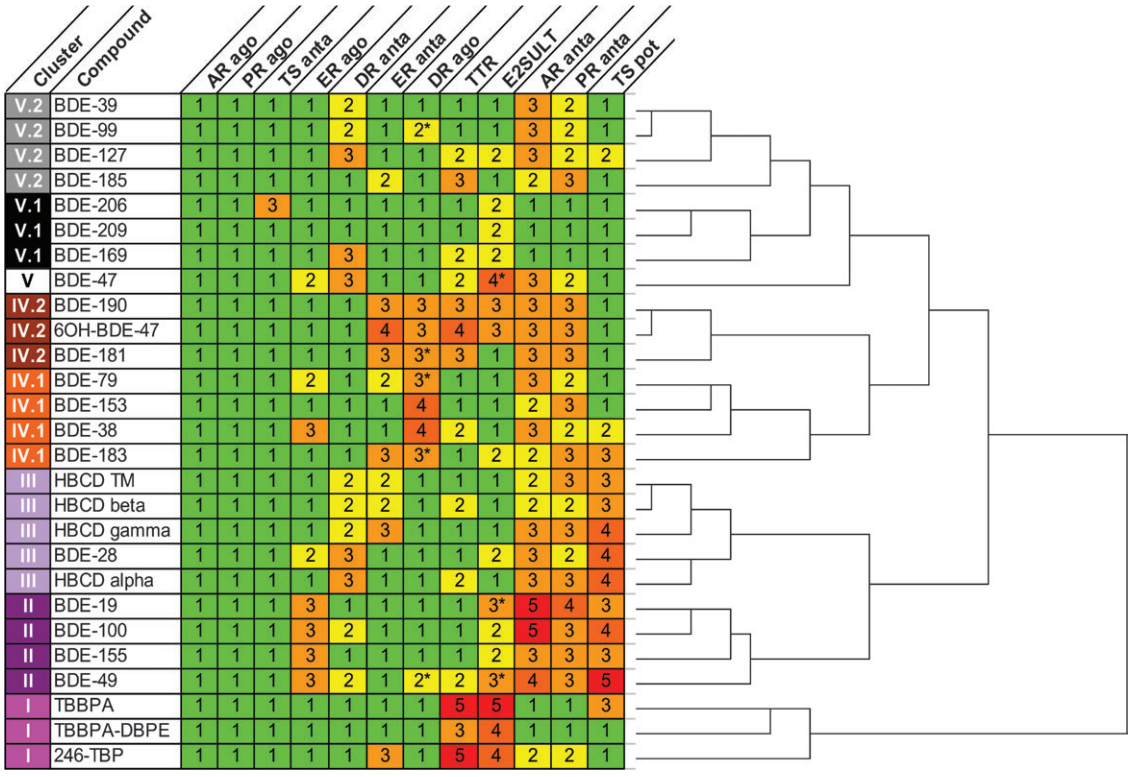

FIG. 3. Hierarchical clustering analysis of the classified *in vitro* toxicity profiles of 27 BFRs. The relative difference in toxicity profiles between two compounds is indicated by the horizontal distances to be bridged when moving from one compound to the other according to the branches of the dendrogram tree shown at the right side. Numbers indicate the relative potencies for the different ED modes of action according to the criteria in Table 3. Asterisks indicate cases with BFR dose-response curves that did not reach the maximum response of reference compounds TCDD (DR ago) or PCP (E2SULT). In these cases, EC_{50} and IC_{50} values were calculated as the point of inflection in the compound-specific dose-response curve; ago, agonism; anta, antagonism; pot, potentiation; other abbreviations as in Figure 2.

confirmed a lack of AR-(ant)agonistic potency recently reported by Kitamura *et al.* (2005).

In addition, PR-antagonistic potencies of BFRs have been demonstrated for the first time. In general, PR-antagonistic potencies were more than three orders of magnitude lower than found for the abortifacient RU-486, of which the mechanism of action is based on blocking PR. Especially for the lower-brominated diphenyl ethers, a significant correlation was found between PR-antagonistic potencies and AR-antagonistic potencies (Table 5), although the first was generally lower than the latter. The PR-antagonistic activities of HBCD and again penta-BDE-100 seem to be especially of interest, given the abundant presence of these BFRs in the environment. The low PR-antagonistic activity of the penta-PBDE mixture (Table 5) may partly be attributed to not only BDE-100 but also to BDE-66, BDE-85, BDE-138, and BDE-154, for which Harju *et al.* (in press) predicted IC_{50} values of 9–20μM using a QSAR model based on the PR-CALUX results presented here. In general, the QSAR model predicted highest PR-antagonistic potency for PBDEs with a tri- or tetra-*ortho* substitution.

In contrast to the DR and ER responses, AR and PR were not activated agonistically by any of the BFRs tested. A lack of the AR-activating potency seems to be a general feature for environmental AR ligands (e.g., Schreurs *et al.*, 2005), possibly because activation of the AR is a more complex mechanism depending on C- and N-terminus interactions and specific coactivators (Sonneveld *et al.*, 2005). Similar complex activation processes may be involved in PR activation.

The T-Screen results confirmed effects of the high-production BFRs TBBPA and HBCD on *in vitro* thyroid hormone–signaling studies described before (Kitamura *et al.*, 2002, 2005; Schriks *et al.*, 2006; Yamada-Okabe *et al.*, 2005). In addition, the current study demonstrated such potencies for another eight PBDE congeners, of which BDE-100 and BDE-49 are the most environmentally relevant. Interestingly, most BFRs exhibited T-Screen activities only when tested in the presence of T3 (Table 8). For HBCD, similar results were obtained before (Schriks *et al.*, 2006; Yamada-Okabe *et al.*, 2005). For TBBPA, however, Kitamura *et al.* (2002, 2005) demonstrated the T3-like activity of TBBPA in GH3 cells in both the absence and the presence of T3. The explanation for these contradictory results could be the use of serum-free medium in our study, excluding the chance of any remaining T3 in the exposure mixture. The use of serum-free medium may also (partially) explain the

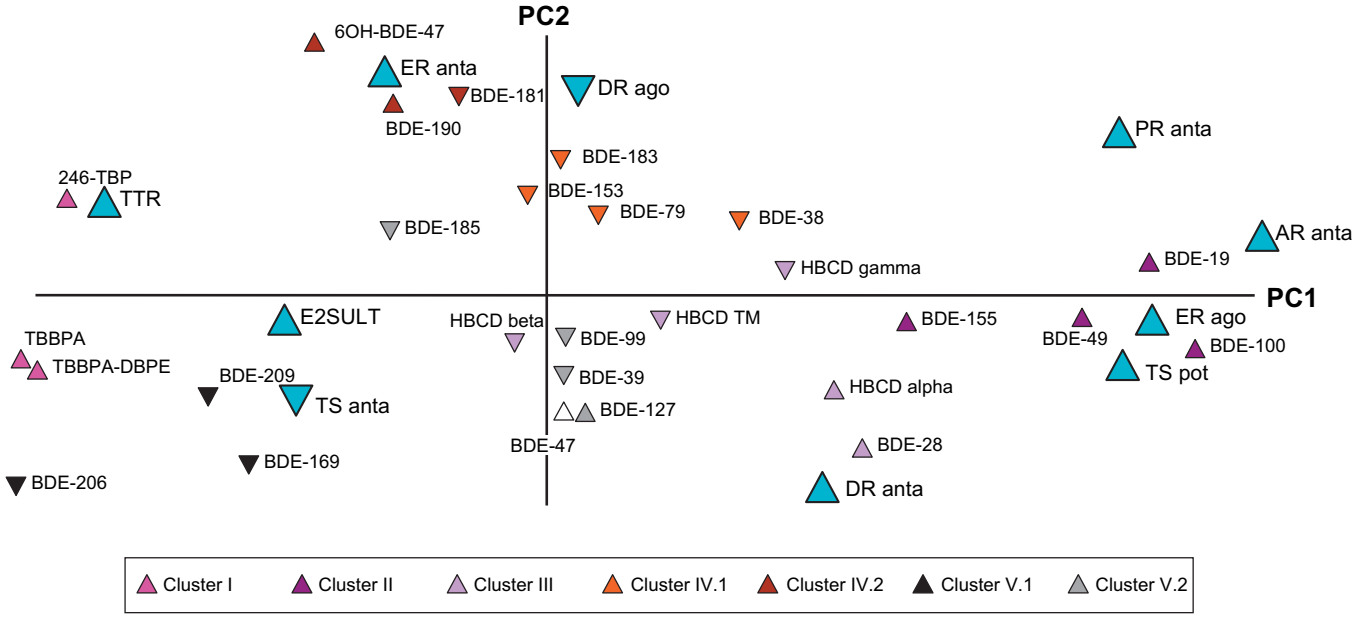

FIG. 4. PCA ordination diagram of the 27 BFR scores (small triangles) with the superimposed loading plot showing the original 10 bioassay responses (large triangles; AR- and PR-agonistic responses have been excluded from PCA analysis). Colors indicate the different clusters distinguished by the hierarchical clustering in Figure 3; for each BFR and bioassay, the triangle points at the direction of ordination in the third dimension (PC3), i.e., above (Δ) or below (∇) the two-dimensional plane described by PC1 and PC2; ago, agonism; anta, antagonism; pot, potentiation; other abbreviations as in Figure 2.

differences in cytotoxicity of TBBPA, which starts at concentrations > 1μM in our study but did not occur until 100μM in the study of Kitamura *et al.* (2005).

In a previous study on the T4-TTR–binding competition by BFRs, Meerts *et al.* (2001) found no TTR binding for 17 different PBDE congeners at maximum concentrations of 0.25μM (including BDE-47, BDE-99, BDE-100, BDE-153, and BDE-190). In the present study, TTR binding of PBDEs was found at much higher concentrations indeed. As in the present study, Meerts *et al.* (2001) also found higher TTR-binding potencies for TBBPA and 246-TBP than for the natural ligand T4 (Table 7). These results confirm the proposed prerequisite for TTR-binding PCBs, i.e., hydroxylation at the *para* position with one, but preferably two, adjacent halogen substituent (Lans *et al.*, 1993). The structure of 6OH-BDE-47 does not fully comply with this optimum prerequisite. This may explain why the TTR-binding potency of this PBDE metabolite is higher than for nonhydroxylated PBDEs but lower than for TBBPA and 246-TBP.

Inhibition of E2SULT is another endocrine-related endpoint that so far was demonstrated for a limited number of BFRs, i.e., 246-TBP, TBBPA, and some OH-PBDE congeners (Kester *et al.*, 2000, 2002). As for TTR binding, Kester *et al.* (2000) found highest E2SULT-inhibiting potency for coplanar *para*-hydroxylated organohalogens with halogen substitution on the phenolic ring, preferably at both places adjacent to the hydroxy group.

Indeed, 246-TBP and TBBPA had high to very high E2SULT-inhibiting potencies (Kester *et al.*, 2002; Table 7), whereas 6OH-BDE-47 (Table 7) had moderate to high inhibiting potencies. Moreover, the E2SULT-inhibiting potency was found for another four PBDEs (Table 7), although the relatively high E2SULT-inhibiting potency of the most common congener BDE-47 (Fig. 3) is probably overestimated because it is based on an IC_{50} value of 0.8μM (i.e., point of inflection in the dose-response curve of Fig. 2). Compared to the reference compound PCP, however, only 40% inhibition was reached at the maximum BDE-47 concentration of 10μM (Fig. 2).

The ER-agonistic potencies found for the environmentally relevant PBDE congeners BDE-28, BDE-47, and BDE-100 (Table 6) confirm ER-CALUX results reported by Meerts *et al.* (2001). Compared to this latter study, estrogenic activity of BDE-99 (only 2% of the maximum induction by 30pM E2) was probably too low to be confirmed in the present study, whereas antiestrogenic potency for BDE-153 and BDE-190 could only be confirmed for the latter congener. The additional ER-agonistic responses found in the present study for BDE-19, BDE-38, BDE-49, BDE-79, and BDE-155 and ER-antagonistic responses for hepta-brominated BDE-181, BDE-183, BDE-185, BDE-190, and the octa-PBDE mixture (Table 6) confirm the general observation by Meerts *et al.* (2001) that estrogenic activity is associated with lower-brominated PBDEs and antiestrogenic activity with higher-brominated PBDEs. For estrogenic PBDEs, Meerts *et al.* (2001) described common structural features as ‘‘two ortho (2,6)-bromine atoms on one phenyl ring, at least one *para*-bromine (preferably on the same phenyl ring as the ortho bromines), and nonbrominated *ortho*-*meta*– or *meta*-carbons on the other phenyl ring.’’ Of the six

PBDEs with moderate ER-agonistic activity (Table 6), only BDE-100 and BDE-155 comply with this structure-activity relationship. The current data suggest that ER-agonistic activity of PBDEs concurs with a [2,2′6] or a [2,2′,4] substitution and no bromine substitutions adjacent to each other. Still, both closely related descriptions do not cover the relatively potent 3,4,5-substituted BDE-38. With respect to non-PBDEs, a weak agonistic ($EC_{50} = 19\mu M$) as well as an antagonistic ($IC_{50} \geq 10\mu M$) activity was found for TBBPA in a luciferase reporter gene assay with human mammary cancer MCF-7 cells (Kitamura *et al.*, 2005), but not in the ER-CALUX assay (Table 6; Meerts *et al.*, 2001). The high antiestrogenic activity for 6OH-BDE-47 and 246-TBP (Table 6) should be interpreted with care. Since this ER-antagonistic activity could not be competed out with 1000pM E2 ($= 250 \times EC_{50}$), it is possible that these compounds suppress the E2-induced response by cytotoxicity, although additional cell viability assays with resazurin did not indicate cytotoxicity for 6OH-BDE-47 or 246-TBP in ER-CALUX cells.

The present study confirms results from earlier DR-CALUX bioassay studies, indicating that PBDEs may act as weak DR agonists. For BDE-28, BDE-47, BDE-99, BDE-100, 246-TBP, and HBCD (alpha, beta, and gamma diastereoisomers), the low to absent DR-agonistic potency (Table 4) confirmed earlier DR-CALUX results (Behnisch *et al.*, 2003; Meerts *et al.*, 1998; Murk *et al.*, 1996). Maximum DR-agonistic potencies of more than four orders of magnitude lower than TCDD were found for BDE-38, BDE-153, and BDE-190 (Table 4), of which the latter two are relevant from an environmental point of view. The DR-agonistic potency of BDE-190 was similar to that reported by Meerts *et al.* (1998) but higher than found by Behnisch *et al.* (2003). BDE-153 and BDE-183 showed a higher DR-agonistic potency compared to all earlier studies (Behnisch *et al.*, 2003; Meerts *et al.*, 1998; Murk *et al.*, 1996). The relatively high DR-agonistic potencies of [2,2′]-substituted BDE-153 and BDE-183 are remarkable results, given the lack of DR-agonistic potency reported for [2,2′]-substituted PCBs including PCB-153 (Van der Plas *et al.*, 1998). Possibly, BDE-153 fits better in the DR-binding pocket than PCB-153 due to the presence of an ether bridge between the two phenyl rings, which is absent in PCB-153.

DR antagonism of BDE-28, BDE-47, BDE-99, and BDE-100 confirmed DR-CALUX results by Meerts *et al.* (1998), but potencies were higher in the present study. In both studies, the environmentally predominant BDE-47 was the most potent congener. In addition, DR-antagonistic responses were found for four other PBDE congeners, the penta- and the octa-PBDE mixtures, and HBCD (Table 4), although potencies were moderate to low.

Except for the commercially obtained PBDEs 19, 38, 39, 79, 127, and 185, all other PBDE congeners and mixtures were cleaned on a charcoal column before preparing stock solutions in DMSO. Cleanup was performed to remove low levels of DR-agonistic polybrominated dibenzofurans (PBDFs) and dibenzo-*p*-dioxins (PBDDs) that may be formed during PBDE synthesis and may act as potent DR agonists. This removal may explain why the present study could not confirm the DR-agonistic potency reported for noncleaned BDE-209 (Behnisch *et al.*, 2003). Although the contribution of PBDF and PBDD contamination to the DR-agonistic response of BDE-38 and BDE-79 (Table 4) is highly unlikely (Accu Standard, personal communication), it cannot be completely excluded (Åke Bergman, personal communication). Therefore, DR-agonistic responses of these two PBDEs should be handled with care. Non-PBDE compounds were not cleaned before preparing stock solutions in DMSO because PBDF and PBDD formation is not to be expected during the synthesis of these compounds. Nevertheless, the TMs of HBCD and TBBPA-DBPE may still be contaminated with other intermediate compounds. In particular, a possible contamination of TBBPA-DBPE with TBBPA could explain the (very) high potency of TBBPA-DBPE in the TTR and E2SULT assays.

Toxicity Profiling

Hierarchical cluster analysis and PCA were used to present the complex data set (i.e., toxic potencies of 27 BFRs towards 12 endocrine-related modes of action) in a single diagram. Both the dendrogram in Figure 3 and the PCA plot in Figure 4 can be used to identify the most potent BFRs and their most important modes of action. By pattern recognition, both methods provide new information about similarity and dissimilarity in toxicity profiles of the individual BFRs, which can be indicative of structural features or other chemical characteristics that are required for a given mode of action. In addition, this new information can be used to design further (*in vivo*) studies and to select indicator BFRs representing different toxicity profiles that should be tested in such studies (see next paragraph).

Cluster II (Fig. 3) for instance consists of tri- to hexa-brominated BDE-19, BDE-49, BDE-100, and BDE-155 with a di-*ortho* [2,2′] substitution and no bromine substitutions adjacent to each other on a single aromatic ring. BDE-47 is the only other BFR in the test set that complies with this description. Although its toxicity profile is very similar to that of BDE-19, BDE-49, BDE-100, and BDE-155 (four out of five endpoints overlap), BDE-47 is not clustered accordingly in cluster II, probably because of its lower potencies. The similarity in profile but dissimilarity in potencies is illustrated in the PCA plot (Fig. 4), where BDE-47 is located in between clusters V.1 (inactive BFRs) and II, indicating that BDE-47 was erroneously clustered with the rather inactive rest BFRs in Figure 3.

For the HBCD TM and the three individual diastereoisomers, common structural features are obvious and are well reflected by a high correspondence in toxicity profiles (Figs: 3 and 4). Apparently, the toxicity profile of mono-*ortho*–brominated BDE-28 resembles the HBCD profile better than

the [2,2′]-substituted PBDEs profile (cluster II), probably due to the lower potency of BDE-28 in the various bioassays than the PBDEs from cluster II. Cluster I contains compounds with toxicity profiles that deviate most from all other BFRs, i.e., moderate to very high potency in the TTR and E2SULT assay (Fig. 3). It contains the two hydroxylated BFRs, TBBPA and 246-TBP, as well as the TBBPA-DBPE TM, which may contain considerable amounts of its phenolic precursor TBBPA. On the other hand, 6OH-BDE-47 is the only hydroxylated compound from the test set that is not clustered in cluster I, probably because its toxic potency was not as restricted to the TTR and E2SULT assays as it was for the BFRs from cluster I. It cannot be excluded, however, that the moderate to high AR-, PR-, and ER-antagonistic potencies of 6OH-BDE-47 are caused by cytotoxicity rather than competition for receptor binding and should therefore be classified as 1 in Figure 3. In that case, the toxicity profile of 6OH-BDE-47 resembles that of the BFRs in cluster I.

Resemblance in structure-activity relationships can also explain the strong correlations between different endpoints. For instance, the correlation between ER-agonistic, AR-antagonistic, and PR-antagonistic potencies (Fig. 4) can be explained by high bioassay responses of BDE-19 and BDE-100, complying with the structural requirements for ER-activation (i.e., low bromination with a [2,2′6] or a [2,2′,4] substitution and no bromine substitutions adjacent to each other), AR inactivation (low bromination with *ortho* or *ortho-para* substitution), and PR inactivation (tri- or tetra-*ortho* substitution) (this study; Harju *et al.*, in press). As pointed out above, the strong correlation between TTR binding and E2SULT inhibition can be attributed to TBBPA and 246-TBP, which comply with the similar structure-activity requirements for these endpoints, i.e., an OH-group with one and preferably two adjacent bromine substituents.

Possible Implications for Human and Environmental Health

The finding that most BFRs tested in this study have considerable antiandrogenic potency is intriguing. The antiandrogenic potency of the environmentally relevant BDE-100 was 13 times higher than that of the well-known antiandrogenic drug flutamide. The presence of antiandrogenic compounds, like BFRs in the aquatic environment might present a plausible alternative explanation for the observed alterations in fish reproductive functions, which have so far been associated with the presence of (xeno-)estrogens in the aquatic environment (e.g., Sumpter, 2005). Exposure of fish to antiandrogens has been associated with gonadal changes (induction of intersex), reduced spermatogenesis, demasculinization, and reduced sperm counts (Bayley *et al.*, 2003; Kiparissis *et al.*, 2003). Similar changes in testicular function and sperm production have been observed in rodents exposed to antiandrogens (Kubota *et al.*, 2003; Mahood *et al.*, 2005; Uzumcu *et al.*, 2004) including the penta-PBDE mixture DE-71 (Stoker *et al.*, 2005). Further studies aim at the elucidation of the relevance of antiandrogenicity by man-made chemicals including BFRs for changes in reproductive functioning in fish. In the same context, the antiprogestagenic properties of a wide set of BFRs should be considered with concern, as Sumpter (2005) recently stressed the extreme importance of some progesterones to fish, in which they act as pheromones crucial for eliciting physiological and behavioral responses in the other sex.

The *in vitro* potency of BFRs to interfere with T3-mediated effects also deserves follow-up investigation, especially since the T3-potentiating effect of HBCD alpha and the T3-antagonistic effect of BDE-206 were confirmed in additional T3-dependent *in vivo* assays with amphibian larvae (Schriks *et al.*, in press). The possibility that these compounds can affect T3-regulated processes in animal development is especially relevant because of their environmental relevance given the bioaccumulating potency of HBCD alpha (Zegers *et al.*, 2005) and the presence of BDE-206 in sediments at relatively high concentrations (LaGuardia *et al.*, 2004).

For hydroxylated BFRs, the *in vivo* consequences of high *in vitro* TTR-binding and E2SULT-inhibiting potency for possible hypothyroidal and estrogenic effects deserves further attention. This holds not only for hydroxylated parent compounds such as TBBPA and 246-TBP but also for hydroxylated metabolites of PBDEs formed by microsomal biotransformation, which have much higher TTR-binding and E2SULT-inhibiting potency than the parent PBDE congener (Hamers *et al.*, in press; Meerts *et al.*, 2000). In addition, direct toxicity of PBDEs also seems to be increased by hydroxylation. Additional experiments confirm that PBDE cytotoxicity to DR- and ER-CALUX cells increases after metabolization, especially for lower-brominated BFRs (Hamers *et al.*, in press).

Based on these new insights, implications for human and environmental health are expected for BFRs grouped in clusters I (TTR-binding and E2SULT inhibition), II (antiandrogenicity and antiprogestagenicity), and III (as II with T3 potentiation). For a more elaborate hazard assessment, we therefore suggest to test the *in vivo* ED potency of TBBPA, BDE-100, and HBCD, which are representative BFRs for clusters I, II, and III, respectively, with high environmental relevance. Special attention should be given to hypothyroidal and estrogenic effects of TBBPA, to estrogenic, antiandrogenic, antiprogestagenic, and hyperthyroidal effects of BDE-100, and to antiandrogenic, antiprogestagenic, and hyperthyroidal effects of HBCD.

CONCLUDING REMARKS

The systematic screening demonstrated *in vitro* ED potencies for a wider set of BFRs and for more modes of action than was ever tested before. Antiandrogenic, antiprogestagenic, (anti-)estrogenic, (anti–)dioxin-like, and T3-antagonistic potencies of BFRs were demonstrated at the receptor level. In

addition, ED potencies of BFRs were revealed that are not directly linked to receptor (in-)activation, namely protein-binding (TTR), enzyme inhibition (E2SULT), and potentiation of T3-mediated effects. For some BFRs, the ED potency was higher than for natural ligands or clinical drugs used as positive controls, viz, AR antagonism by di-*ortho*-[2,2′]-substituted PBDEs with no bromine substitutions adjacent to each other, and T4-TTR competition and E2SULT inhibition by hydroxylated BFRs. Based on similarity in ED profiles, BFRs could be classified into five different clusters. TBBPA, BDE-100, and HBCD were identified as the most environmentally relevant BFRs representing different toxicity profiles. These findings support further investigation of the potential ED effects of these BFRs in man and wildlife.

ACKNOWLEDGMENTS

This study was carried out within the EU-supported program FIRE (EU contract no. QLK4-CT-2002-00596; RIVM 2005). We thank Prof. Dr Åke Bergman and Dr Göran Marsh (Stockholm University) for supplying most BFR standards and Prof. Dr Hansruedi Glatt (German Institute of Human Nutrition) for supplying recombinant human SULT1E1 enzyme.

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