In Vitro Estrogenicity of Polybrominated Diphenyl Ethers, Hydroxylated PBDEs, and Polybrominated Bisphenol A Compounds

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Polybrominated diphenyl ethers (PBDEs) are used in large quantities as additive flame retardants in plastics and textile materials. PBDEs are persistent compounds and have been detected in wildlife and in human adipose tissue and plasma samples. In this study, we investigated the (anti)estrogenic potencies of several PBDE congeners, three hydroxylated PBDEs (HO-PBDEs), and differently brominated bisphenol A compounds in three different cell line assays based on estrogen receptor (ER)-dependent luciferase reporter gene expression. In human T47D breast cancer cells stably transfected with an estrogen-responsive luciferase reporter gene construct (pERE-tata-Luc), 11 PBDEs showed estrogenic potencies, with concentrations leading to 50% induction (EC₅₀) varying from 2.5 to 7.3 µM. The luciferase induction of the most potent HO-PBDE (2-bromo-4-(2,4,6-trimethoxy)phenol) exceeded that of estradiol (E₂), though at concentrations 50,000 times higher. As expected, brominated bisphenol A compounds with the lowest degree of bromination showed highest estrogenic potencies (EC₅₀ values of 0.5 µM for 3-monobromobisphenol A). In an ERα-specific, stably transfected human embryonic kidney cell line (293-ERα-Luc), the HO-PBDE 4-(2,4,6-trimethoxy)phenol was a highly potent estrogen with an EC₅₀ < 0.1 µM and a maximum 35- to 40-fold induction, which was similar to E₂. In an analogous ERβ-specific 293-ERβ-Luc cell line, the agonistic potency of the 4-(2,4,6-trimethoxy)phenol was much lower (maximum 50% induction compared to E₂), but EC₅₀ values were comparable. These results indicate that several pure PBDE congeners, but especially HO-PBDEs and brominated bisphenol A analogs, are agonists of both ERα and ERβ receptors, thus stimulating ER-mediated luciferase induction in vitro. These data also suggest that in vivo metabolism of PBDEs may produce more potent pseudostrogens. Key words: ER-CALUX, estrogenicity, flame retardants, hydroxylated compounds, polybrominated diphenyl ethers. Environ Health Perspect 109:399–407 (2001). [Online 27 March 2001] http://ehpnet1.nih.gov/docs/2001/109p399-407meerts/abstract.html

Polybrominated diphenyl ethers (PBDEs) are widely used as additive flame retardants in many different polymers, resins, and substrates at concentrations ranging from 5% to 30% (1). Because of the widespread production and use of PBDEs, their high binding affinity to particles, and their lipophilic characteristics, several PBDE congeners bioconcentrate and bioaccumulate in the environment in a manner similar to the structurally related polychlorinated biphenyls (PCBs) (1–3). PBDEs have been detected in various biotic samples such as birds, seals, whales, and even in human blood, adipose tissue, and breast milk (4–10). The congeners 2,2′,4,4′-tetrabDE (BDE-47), 2,2′,4,4′,5-pentaBDE (BDE-99), and 2,2′,4,4′,6-pentaBDE (BDE-100) are generally the dominant congeners found in wildlife and humans. The relevance of PBDEs as environmental contaminants has been demonstrated by their accumulation in human breast milk, where concentrations in Swedish women have increased over the last 2 decades from 0.07 ng/g lipid weight in 1972 to 4.02 ng/g lipid weight in 1998 (8).

Although PCB concentrations in wildlife are still higher than PBDE concentrations, they are declining over the same time period. The most sensitive end points of PBDE toxicity in vivo are effects on thyroid function, observed as induction of thyroid hyperplasia and alteration of thyroid hormone production (i.e., lowering of free and total thyroxine (T₄) concentrations) in rats and mice (11,12). Consistent with these findings is the recent observation that several PBDE congeners were able to displace T₄ from transthyretin (TTR, a plasma transport protein of thyroid hormones) in vitro, after metabolic conversion to hitherto unidentified metabolites (13). These phenomena have also been observed for other organohalogen compounds such as PCBs and their hydroxylated metabolites (14,15, and references therein).

Another property that PBDEs share with PCBs and the polybrominated biphenyls (PBBs) is the dioxinlike, Ah receptor-mediated induction of cytochrome P450 1A1 and 1A2 in vitro (16) and in vivo (17). Recently we demonstrated by means of an Ah receptor-mediated, chemically activated luciferase expression cell line (the Ah-CALUX-assay) (18–20) that several pure di- to hepta-brominated PBDE congeners were able to act via this Ah receptor pathway in vitro as agonists and antagonists in a congener-specific manner (21). For example, 2,3,4,4′,5,5′-hexaBDE (BDE-166) and 2,3,4,4′,5,5′-hexaBDE (BDE-190) were relatively strong Ah receptor agonists with potencies comparable to the mono-ortho 2,3,3′,4,4′-pentacB (CB-105) and 2,3,4,4′,5-pentaB (CB-118) (22).

Some studies have indicated that hydroxylated PBDEs (HO-PBDEs) are of potential environmental importance. In liver microsomes of rats, several PBDE congeners were biotransformed to metabolites (13). Öh and Klasson-Wehler (23) demonstrated that 2,2′,4,4′-tetrabDE (BDE-47) is biotransformed to HO-PBDEs in rats and mice. 3,5-Dibromo-2-(2,4-dibromophenoxy)phenol is a hydroxy-BDE that has been identified in blood plasma of Baltic salmon (24) at levels similar to those of the major PBDE congeners. Information on the endocrine activity of hydroxylated PBDEs is presently limited to the ability of several HO-PBDEs to bind competitively to the thyroid hormone receptor (25) and to TTR (13).

Studies showing that many industrial chemicals are weakly estrogenic compared to natural estrogens (26–28) have raised concern about their safety. For example, o,p′-DDT, bisphenol A, nonylphenol, and various phthalates possess estrogenic activity (27). The presumption is that these xenosterogens may disrupt normal endocrine function, which can lead to reproductive failure.
and cancer of estrogen-sensitive tissues in humans and wildlife (29). Antiestrogenic activity by anthropogenic compounds has received less attention (30). Although the inhibition of hormone action and the resulting toxicological consequences have not been demonstrated conclusively, antiestrogenic action could critically affect sensitive reproductive and developmental processes as well (30). To date there have been no reports investigating the (anti)estrogenic activities of PBDEs and HO-PBDEs.

The aim of this study was to determine the (anti)estrogenic activity of 17 PBDE congeners. We also examined three hydroxylated PBDEs that have halogen substitution patterns similar to those of thyroid hormones. The (anti)estrogenic activity of these compounds was tested in vitro, using an estrogen-responsive luciferase reporter cell line (T47D.Luc) (31). We compared the structure–activity relationships for (anti)estrogenicity of PBDE and HO-PBDE congeners with numerous other brominated flame retardants, such as differently brominated bisphenol A compounds. We also tested the (anti)estrogenic activity of the most potent PBDEs and HO-PBDEs.

Materials and Methods

**Chemicals.** The 17 PBDE congeners (>98% pure; Figure 1, Table 1) were synthesized as described earlier (35,36). Three HO-PBDEs, 4-(2,4,6-tribromophenoxy)phenol (T₂-like HO-BDE), 2-bromo-4-(2,4,6-tribromophenoxy)phenol (T₃-like HO-BDE), and 2,6-dibromo-4-(2,4,6-tribromophenoxy)phenol (T₄-like HO-BDE) (Figure 1) were synthesized as described by Marsh et al. (25) and were at least 99% pure. We use the abbreviations for these HO-PBDEs (T₂-like, T₃-like, T₄-like HO-BDE) according to their resemblance in halogen substitution patterns to the thyroid hormones 3,5-diiodothyronine (3,5-T₂), 3,3',5-triiodothyronine (T₃), and 3,3',5,5'-tetraiodothyronine (T₄). The core structure of PBDEs and the structures of the HO-PBDEs used in this study are shown in Figure 1, including the structure of the analog 4-phenoxyphe nol. The numbering system for individual PBDE congeners is based on the numbering system applied to PCBs (37). 4-Phenoxyphe nol and bisphenol A were obtained from Aldrich Chemical Company (Bornem, Belgium), 17β-Estradiol (E₂; 99%) and ethanol (100%, pro analysis) were purchased from Sigma Chemical Company (St. Louis, MO, USA). ICI 182,780 was a gift from A. Wakeling, Zeneca Pharmaceuticals (Macclesfield, Cheshire, U.K.). 3-Monobromobiphenyl A (MBBPA; 96.5% pure, with 3.5% 3,3′-dibromobiphenyl A), 3,3′-dibromobiphenyl A (dBBPA; 99.4% pure, with 0.6% 3,3′,5-tribromobiphenyl A), and 3,3′,5-tribromobiphenyl A (triBBPA; 100% pure) were synthesized by bromination of biphenyl A using bromine in acetic acid at room temperature. The test chemicals and E₂ were dissolved in ethanol or dimethyl sulfoxide (D M SO; 99.9% pure, Janssen Chimica, Geel, Belgium) for use in the in vitro assays.

**Cell culture.** We used the human T47D breast cancer cell line stably transfected with an estrogen-responsive luciferase reporter gene construct (pEREtata-Luc) (31) to study the in vitro (anti)estrogenic activity of PBDEs and HO-PBDEs. The T47D.Luc cells were cultured in a 1:1 mixture of Dulbecco's Modified Eagle's (DMEM) medium and Ham's F12 (DF) medium (Gibco BRL, Life Technologies, Breda, The Netherlands) supplemented with sodium pyruvate, and 7.5% fetal calf serum (heat inactivated) at 37°C and 7.5% CO₂.

The preparation of the stably transfected 293-Luc cell lines (ERα and ERβ) has been described in detail elsewhere (32). Briefly, human 293 embryonal kidney (HEK) cells (ATCC, American Type Culture Collection, Rockville, MD, USA) were stably transfected with the pEREtata-Luc construct (31,32) cotransfected with an antibiotic resistance gene. This cell line was subsequently transfected with a recombinant human estrogen receptor (ERα or ERβ) cDNA and the luciferase reporter gene construct (32–34).

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Structure of PBDEs, the three hydroxylated PBDEs, 4-phenoxyphe nol, and the differently brominated biphenyl A analogs. The hydrogens have been omitted for clarity.
human estrogen receptor (ERα or a short
form of ERβ, ERβ-s) cDNA and a different
antibiotic resistance gene. The 293-ERα-
and 293-ERβ-s-Luc cell line cultures were
lined in a 1:1 mixture of D M E M and DF
medium supplemented with 7.5% fetal calf
serum (heat inactivated) at 37°C and 7.5%
CO2.

**ER-CALUX assay.** We performed the
T47D.Luc-based assay as described previ-
ously (31). The cells were trypsinized, resus-
pended in assay medium, and seeded in
96-well plates (Packard, Meriden, CT, USA)
at a density of 5,000 cells per well in 100 µL.
The assay medium consisted of phenol red-
dee-free DF and fetal calf serum treated with 5%
dextran-coated charcoal (DCC-FCS). DC F-
CFS was prepared as described by Horwitz
and McGuire (38). After 24 hr, when cells were
approximately 50% confluent, the assay
medium was renewed. After another 24 hr,
the assay medium was replaced by incuba-
tion medium (for preparation, see below),
containing DM SO or ethanol stock
solutions of the test compounds or estradiol.
Solvent concentrations did not exceed 0.1%.
The incubation medium was removed after
an incubation of 24 hr at 37°C in an atmos-
phere of 7.5% CO2. Cells were washed twice
with 100 µL phosphate-buffered saline
(PBS) and subsequently lysed in 30 µL low
salt (LS) buffer containing 10 mM Tris (pH
7.8), 2 mM dithiothreitol (DTT), and 2
mM ¹,2-diaminocyclohexane-N,N,N’,N’-
tetraacetic acid. After 10 min of incubation
on ice, the 96-well plates were frozen at
–20°C for a minimum of 30 min and maxi-
mum of 1 day to lyse the cells. The plates
were thawed on ice and shaken for 5 min at
room temperature. We measured luciferase
activity in a luminometer (Labsystems
Luminoscan RS, Breda, The Netherlands)
with automatic injection of 100 µL flash mix
(pH 7.8) per well containing 470 µM
luciferin, 20 mM tricine, 1.07 mM
(MgCO3)3, M g (OH )2, 5 H 2 O, 2.67 mM
MgSO4, 2.5 mM EDTA, 5 mM ATP, and 2
mM DTT (pH 7.8).

293-ERα and 293-ERβ-s Luc assay. The
293-ERα- and 293-ERβ-s-Luc-based assays
were performed similarly to the ER-CALUX
assay and have been described previously
(32–34). Briefly, cells were trypsinized and
resuspended in assay medium composed of
phenol red-free DF containing 30 mM selenium,
10 µg/mL transferin, and 0.2% BSA
supplemented with 5% D C C-FCS. The
cells were seeded in 96-well plates at a den-
sity of 15,000 cells per well in 200 µL assay
medium. After 48 hr the cells were 50–60%
confluent, and the assay medium was
replaced by incubation medium (i.e., con-
taining a 1,000-fold dilution of test com-
ounds) as described for the ER-CALUX
assay. After an incubation of 24 hr at 37°C
in an atmosphere of 7.5% CO2, the plates
were transferred to ice and the medium was
removed by suction. Luciferase production
was assayed as described above for the ER-
CALUX assay.

**Exposure of cells.** Before the T47D.Luc
cell incubations, the PBDE and H O-PBDE
stock solutions (prepared in D M SO) and
the brominated bisphenol compounds (pre-
pared in ethanol) were diluted 1,000-fold
in assay medium in a 48-well plate (to obtain
a solvent-concentration of 0.1%/v/v)
and thoroughly shaken, and 100 µL was
added to the cells in 96-well plates. The
nominal concentrations of the toxicants in
the medium were 0.05, 0.1, 0.5, 1.0, and 5 µM,
and for potent compounds concentrations
of 2.5 and 10 µM were also included. For
each experiment, we included a complete E2
standard curve (1–100 pM, 7 different
concentrations in total). In addition, we tested
three calibration points (0, 10, and 30 pM
E2) on every 96-well plate within an experi-
ment.

For the 293-ERα- and 293-ERβ-Luc assays,
the D M SO stock solutions of the
tested compounds were diluted 1,000-fold
in the appropriate assay medium. The nomi-
nal concentrations of the toxicants exposed
to the cells were 1.0, 5.0, and 10 µM. For
each experiment a complete E2 standard
curve (0.001–10,000 pM in eight different
concentrations) was included. For all three
ER-CALUX assays, we tested every toxicant
concentration in triplicate and repeated each
assay at least twice.

**Antiestrogenic effects.** We tested the pos-
sible antiestrogenic effects of the com-
ounds in the ER-CALUX assay at the same
nominal concentrations as for the estrogenic
activity screening. The T47D.Luc cells were
concomitantly with an E2 concentration of
10 pM. This E2 concentration was the approx-
imate EC50 for the induction of luciferase
activity (31). The percentage (v/v) of
DM SO present during these antiestrogenic
incubations was 0.2%. An antiestro-
genic effect in this assay was defined by the
capacity of a chemical to inhibit the
luciferase activity induced by the approxi-
mate EC50 concentration of E2. The per-
cent inhibition is calculated according to
the equation:

\[
\text{Inhibition} = 1 - \frac{\text{EC50 test compound}}{\text{EC50 E2}}
\]

where EC50 test compound is the dose of
the test compound that inhibits 50% of the
maximum luciferase activity of E2.
\[ I(\%) = 100 \left(1 - \frac{L_{\text{test}} - L_{\text{control}}}{L_{E2} - L_{\text{control}}} \right) \quad [1] \]

where \( I \) is the percent inhibition, and \( L_{\text{test}} \), \( L_{\text{control}} \), and \( L_{E2} \) are the average luciferase activity of three test wells, three control wells and six wells incubated with 30 pM of E2, respectively. Using Equation 1, a compound without antagonistic activity will show the same luciferase induction as 10 pM of E2, [i.e., 63.3 ± 7.5% (see “Results”)]. On each plate a positive control of 10 nM of the competitive ER antagonist ICI 182,780 was included in triplicate. ICI 182,780 produces virtually total antagonism of E2-induced luciferase activity at this concentration [i.e., activity measured is equal to solvent control levels (31)].

**Cytotoxicity.** We measured possible cytotoxic effects of the tested compounds in the bioassays using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide activity (39). To determine cytotoxic effects, we seeded cells and exposed them to the test compounds in the same manner as outlined in their corresponding assay procedures.

**Dose-response curves and statistics.** Possible dose-response relations were described by the sigmoidal function

\[ y = a_0 + a_1 / \left[1 + \exp \left( \frac{(a_2 - x)}{a_3} \right) \right] \quad [2] \]

using SlideWrite Plus 4.0 (Advanced Graphics Software, Carlsbad, CA, USA), where \( y \) is the induction of luciferase activity compared to controls for estrogenic effects, or inhibition \( I(\%) \), Equation 1] for anti-estrogenic effects, \( x \) is the logarithm of the dose, and \( a_1 \) is the maximum y-value. We tested the significance of the data fits using one-way analysis of variance at \( p < 0.05 \).

**Results**

**Cytotoxicity**

In the concentration range of 0.01–10 µM, none of the incubations of the PBDEs or HO-PBDEs showed any significant effect on MTT activity relative to the solvent control (data not shown). Furthermore, no cytotoxic effect could be observed by microscopic examination in this concentration range.

**Figure 2.** Estrogenic activity of PBDEs in the T47D.Luc cells. Luciferase induction (%) relative to the maximum induction by E2 (30 pM) after 24-hr exposure to several concentrations of (A) BDE-28, -51, -75, -85 and -119, and (B) BDE-30, -32, -47, -71, and -100. Points are means (\( n = 3 \) ± SD (bars) for each concentration.

**Figure 3.** The estrogenic activity of hydroxy-PBDEs in the T47D.Luc cells. Luciferase induction (%) relative to the maximum induction by E2 (30 pM) after 24-hr exposure to several concentrations of T2-like HO-BDE [4-(2,4,6-tribromophenoxy)phenol], T3-like HO-BDE [2-bromo-4-(2,4,6-tribromophenoxy)phenol], T4-like HO-BDE [2,6-dibromo-4-(2,4,6-tribromophenoxy)phenol], and 4-phenoxyphenol. Points are means (\( n = 3 \) ± SD (bars) for each concentration.

**Figure 4.** The estrogenic activity of differently brominated bisphenols in the T47D.Luc cells. Luciferase induction (%) relative to the maximum induction by E2 (30 pM) after 24-hr exposure to several concentrations of bisphenol A (BPA), monobromobisphenol A (mono), dibromobisphenol A (di), tribromobisphenol A (tri), and tetrabromobisphenol A (teta). Points are means (\( n = 3 \) ± SD (bars) for each concentration.
PBDE concentrations could not exceed 10 µM because of solubility problems and slight cytotoxic effects (data not shown).

**ER-CALUX Assay Based on T47D.Luc Cells**

**Estrogenic effects.** Seventeen PBDE congeners and 3 HO-PBDEs were tested in the T47D.Luc-based ER-CALUX assay for their estrogenic and/or antiestrogenic properties. Eleven PBDEs exhibited luciferase induction (Table 1) in a dose-dependent manner (Figure 2). The most potent PBDE congeners [2,2’,4,4’,6-pentaBDE (BDE-100), 2,4,4’,6-tetraBDE (BDE-75), 2,2’,4,6-tetraBDE (BDE-51), 2,4,4’,6,6’-pentabDE (BDE-119)] showed EC50 values within a small concentration range of 2.5 to 3.9 µM (Table 1). These PBDE agonists were 250,000–390,000 times more potent than the natural ligand, E2.

The T4-like HO-BDE compound demonstrated no estrogenic effect up to 10 µM (Figure 3). In contrast, the T3-like HO-BDE demonstrated no estrogenic effect up to 100 µM (Figure 4). The most potent PBDE agonists (250,000–390,000 times) were 2,3,3’,4,4’,5,6-hepta-BDE (BDE-166), and 2,3,3’,4,4’,5,6-hepta-BDE (BDE-190), which did not induce luciferase activity alone (up to 10 µM, Table 1), reduced E2-induced luciferase activity (Table 2). Moreover, these three PBDE congeners inhibited the E2-induced activity in a dose-dependent manner (Figure 5).

**293-ERα- and 293-ERβs-293-Luc Cell Lines**

As in previous findings (32–34), the luciferase activity for the 293-ERα-Luc assay was more sensitive and responsive to E2 than the 293-ERβs-Luc assay (data not shown). In the present study, the 293-ERα-Luc assay had a 35-fold maximum induction relative to control, which was reached at about 100 pM E2. The lowest observed effect concentration (LOEC) and EC50 for E2 in the 293-ERα-Luc assay were 2.6 pM and 11.9 pM, respectively. In the 293-ERβs-Luc assay, a 16-fold maximum induction was attained at about 1,000 pM E2. The LOEC was 15.3 pM and the EC50 was 90.2 pM for E2.

**Table 2.** Antiestrogenic activity of PBDEs and HO-PBDEs in combination with 10 pM E2 in the ER-CALUX assay with T47D.Luc cells.

| Compound | Bromine substitution | Concentration (µM) | EC50 (µM) | Percent luciferase induction relative to maximum E2 (30 pM) |
|----------|----------------------|-------------------|----------|-----------------------------------------------------|
| Estradiol |                      | 1.0 × 10⁻³        | —        | 63.3 ± 7.5                                           |
| ICI 182,780 |                     | 0.01              | 1.0 × 10⁻³ | 0 ± 1.2                                              |
| PBDEs |                      |                   |          |                                                     |
| BDE-15 | 4,4’                 | 5                 | —        | 62.1 ± 1.5                                           |
| BDE-28 | 2,4,4’               | 0.5               | 5.0      | 81.1 ± 12.9                                          |
| BDE-30 | 2,4,6                | 0.5               | 5.0      | 111.5 ± 3.3                                          |
| BDE-32 | 2,4,6                | 0.5               | 5.0      | 47 ± 10                                              |
| BDE-47 | 2,2’,4,4’            | 0.5               | 5.0      | 106.8 ± 4.0                                          |
| BDE-51 | 2,2’,4,6’            | 0.5               | 5.0      | 75.0 ± 4.8                                           |
| BDE-71 | 2,3’,4,6’            | 0.5               | 5.0      | 108.0 ± 8.2                                          |
| BDE-75 | 2,4,4’               | 0.5               | 5.0      | 52 ± 6                                               |
| BDE-77 | 3,3’,4,4’            | 5.0               | 5.0      | 14.9 ± 7.6                                           |
| BDE-85 | 2,2’,3,4,4’          | 5.0               | 5.0      | 55.1 ± 12.6                                          |
| BDE-99 | 2,2’,4,4’            | 5.0               | 5.0      | 64.9 ± 2.2                                           |
| BDE-100| 2,2’,4,6’            | 0.5               | 5.0      | 68.9 ± 2.9                                           |
| BDE-119| 2,3’,4,6’            | 0.5               | 5.0      | 82.8 ± 2.2                                           |
| BDE-138| 2,2’,3,4,5,5’        | 5.0               | 5.0      | 108.0 ± 15.4                                         |
| BDE-153| 2,2’,4,4’,5’         | 0.5               | 3.1      | 102.6 ± 5.4                                          |
| BDE-166| 2,3,4,4’,5,6         | 0.5               | 0.8      | 95.0 ± 4.8                                           |
| BDE-190| 2,3’,3’,4,4’,5,6,8    | 0.5               | 5.0      | 74.1 ± 5.8                                           |
| HO-PBDEs |                      |                   |          |                                                     |
| 4-Phenoxyphenol |                | 0.5               | —        | 67.0 ± 9.6                                           |
| T2-like HO-BDE |              | 0.5               | 5.0      | 209.3 ± 25.7                                         |
| T3-like HO-BDE |                | 0.5               | 5.0      | 169.4 ± 14.8                                         |
| T4-like HO-BDE |                | 0.5               | 5.0      | 178.5 ± 13.1                                         |

*Concentration at which the induction of luciferase activity by E2 (EC50 concentration of 10 pM) is inhibited by 50%.*

The luciferase activity induced by the test compound and E2 (EC50 concentration of 10 pM) as a percentage of the maximum activity (E2, 30 pM).

The most potent xenoestrogens in the ER-CALUX—BDE-30, BDE-100, 4-phenoxyn-phenol, and T2-like HO-BDE—were investigated for estrogenicity in the 293-ERα- and ERβs-Luc-based assays. Relative to the E2 maximum luciferase induction, the induction of the highest concentration of BDE-30 (10 µM) in the 293-ERα-Luc and 293-ERβs-Luc cell lines (34.2 ± 2.2% and 7.8 ± 3.1%, respectively; Figure 6A,B) was much lower compared to the T47D.Luc cell line (114 ± 31%). At the same concentration,
BD-E-100 showed an induction < 2% in the 293-ERβ-Luc assay, whereas the 293-ERα-Luc assay was more responsive (about 20% relative induction). However, EC50 values of BD-E-30 and BD-E-100 in the 293-ERα-Luc assay (<5.0 µM) and the ER-CALUX assay (3.4 and 2.5 µM, respectively) are comparable.

The T2-like HO-BDE induced maximum response in the 293-ERα-Luc cells to the maximum induction by E2 (30 pM) after 24-hr exposure to several concentrations of BD-E-30 (2,3,4,4’,5,5´-hexaBDE), BD-E-166 (2,3,4,4´,5,6-hexaBDE), and BD-E-190 (2,3,3´,4,4´,5,6-heptaBDE), in the presence of 10 pM E2 (with luciferase induction of 63.3 ± 7.5% of the maximum induction). Points are means (n = 3) ± SD (bars) for each concentration.

Figure 5. The antiestrogenic activity of PBDEs in the T47D.Luc cells. The luciferase induction (%) relative to the maximum induction by E2 (30 pM) after 24-hr exposure to several concentrations of BD-E-153 (2,2’,4,4’,5,5´-hexaBDE), BD-E-166 (2,3,4,4´,5,6-hexaBDE), and BD-E-190 (2,3,3´,4,4´,5,6-heptaBDE), in the presence of 10 pM E2 (with luciferase induction of 63.3 ± 7.5% of the maximum induction). Points are means (n = 3) ± SD (bars) for each concentration.

Discussion

In this study we investigated both the estrogenic and antiestrogenic activity of several PBDE congeners, three hydroxylated PBDEs, and some brominated bisphenol A compounds in vitro. To our knowledge, no studies have been performed on the agonistic or antagonistic activity of these compounds in vivo or in vitro at the level of the estrogen receptor. Of the 17 selected PBDEs, 11 congeners were able to exert estrogenic activities in T47D.Luc cells at LOECs as low as 0.05 µM, and EC50 values ranging from 2.5 to 7.3 µM. In the same ER-CALUX assay, the organochlorine pesticides methoxychlor, endosulfan, and chlordane had a similar potency for luciferase induction, about 1.0 × 10⁻⁶ times the potency of E2 (31).

PBDE congeners with the highest estrogenic activity in the T47D.Luc cells were 2,2´,4,4´,6-pentaBDE (BD-E-100), 2,4,4´,6-tetraBDE (BD-E-75), and 2,2´,4,6´-tetraBDE (BD-E-51). BD-E-100 in particular has often been reported among the more common PBDEs found in humans and other mammals (2,7,9). The common structural features among the estrogenic PBDEs are two ortho (2,6)-bromine atoms on one phenyl ring, at least one para-bromine atom (preferably on the same phenyl-ring as the ortho bromines), and nonbrominated ortho-meta or meta carbons on the other phenyl ring. This structure-activity relationship resembles the one suggested by Korach et al. (40) for hydroxylated PCBs in a competitive binding assay, where congeners with the highest binding affinity for the estrogen receptor contained an unsubstituted phenol ring with a p-hydroxy group (e.g., 4-hydroxy-2,4,6-triCB). However, in the case of the brominated diphenyl ethers, the
para position is occupied by a bromine atom. In addition, Connor et al. (41) observed that hydroxy-PCB congeners having one or no chlorine atoms ortho to the para-hydroxy group and 2,4,6-trichlorination on the nonphenolic ring induced luciferase activity from 20 to 60% (at 5 µM) of the E2 maximum induction in Hela Luc cells. Introduction of a single 2- or 3-chloro substituent into the phenolic ring significantly decreased the estrogen potency (e.g., EC50 value of 2,4,4',6-tetraBDE (2.9 µM) is lower compared to 2,3,4,4',6-pentaBDE (3.9 µM)), whereas the introduction of a single 2-bromine (ortho) substituent next to the para-bromine on the nonphenolic ring increased the estrogen potency, though not significantly (EC50 value of 2,2',4,4',6-pentaBDE: 2.5 µM).

Though not vary likely, hydroxylated PBDE metabolites formed in situ may have been involved in the estrogenic effects of the PBDEs in the T47D Luc cells. T47D Luc cells possess some metabolic capabilities such as cytochrome P450-mediated hydroxylation of estrogens and xenobiotics. P450 1A (42,43), P450 1B (43), and 17β-hydroxysteroid dehydrogenase (44) have been reported in T47D Luc cells. However, few data are available on the metabolism of PBDEs, and in the only two studies known reporting PBDE metabolism, the major compound excreted was the parent PBDE. Örn and Klaassen (23) detected five hydroxylated PBDE metabolites (by GC/MS analyses) in feces and various tissues of rats and mice dosed orally with 2,2',4,4'-tetrabDE (BDE-47), but the major compound excreted was BDE-47. Larsen et al. (45) reported a low (about 1% of the total given dose) biliary and urinary excretion of possible metabolites of 2,2',4,4',5-pentaBDE (BDE-99) in conventional and bile-duct cannulated rats.

The hydroxylated PBDE congeners tested in our study have structural resemblance with the thyroid hormones 3,5-diiodothyronine (T3), 3,3',5-triiodothyronine (T3), and 3,3',5,5'-tetraiodothyronine (thyroxine, T4). These HO-PBDEs have been reported to bind to the human α- and β-thyroid hormone receptor (THR) (25) and compete with the natural hormone T4 for binding to a human thyroid hormone transport protein, transthyretin (13) in vitro. Several interactions between the thyroid hormone receptor- and estrogen receptor-mediated pathways have been reported, affecting testis development (46) and behavior (47). Since the structure of the hydroxyphenyl ring in compounds interacting with the ER and THR (hydroxylated PCBs, hydroxylated PBDEs) is similar (with differences in halogen substitution), it is interesting to study the possible interaction of compounds with both pathways. The ranking of estrogenic potency in the T47D Luc cells of the thyroid hormone-like HO-PBDEs was T2-like HO-PBDE (EC50, 0.1 µM) > T3-like HO-PBDE (EC50, 0.5 µM) >> T4-like HO-PBDE. The potencies of the T2-like HO-PBDE and T3-like HO-PBDE were virtually the same as the potency of the phenolic industrial chemicals, such as bisphenol A (0.3 µM (this study), 0.8 µM (31)) and nonylphenol (0.3 µM (31)) tested in the same ER-CALUX assay. Bisphenol A is one of several well-defined phenolic environmental estrogens that are known to elicit estrogen-mediated responses in vivo and in vitro such as the increased proliferation of MCF7 human breast cancer cells (48–51). The ranking order for estrogenicity of the hydroxylated PBDEs (Figure 3) was the reverse order found for binding to the human α- and β-thyroid hormone receptor (THR) (25) and human transthyretin (TTR) (13) in vitro. This comparison between ER and THR interactions emphasizes that nonphenolization of the brominated ring is necessary for optimum interaction with the ER, which was also found for HO-PCBs (40,41). Conversely, like the interaction of the natural iodine-containing T2, T3, and T4 thyroid hormones with THR and TTR, increasing bromination in adjacent positions on the HO-PBDE increases THR and TTR binding affinity. The same is true for the brominated bisphenols. The ranking of estrogenic potency in the T47D Luc cells of the brominated bisphenols was monoBBA (EC50, 0.5 µM) ~ diBBA (EC50, 0.3 µM) >> triBBA (EC50, > 10 µM) >> T4BBA, and was also the reverse order found for interaction with human TTR in vitro (13). The addition of bromine atoms in the meta position of the aromatic ring (in diBBA) had no significant effect on the estrogenic potency. This is in line with results published by Perez et al. (51), where the estrogenicity of 2,2-bis(4-hydroxy-3-methylphenyl)propane (i.e., one methylgroup in the meta position of one aromatic ring) in a bioassay with MCF7 human breast cancer cells was not changed compared to bisphenol A. However, the introduction of two bromine atoms in the meta position of one aromatic ring drastically decreased the estrogenic potency (triBBA, this study).

In contrast to the HO-PBDEs, the major HO-PCBs identified in human serum were mostly antiestrogenic but exhibited low to nondetectable estrogenic activities in several in vitro bioassays (48). At concentrations as high as 10 M, several 4-OH-substituted PCBs were not estrogenic toward binding of rat uterine ER. Furthermore, the same HO-PCBs did not induce the proliferation of MCF7 human breast cancer cells, or the luciferase activity of transiently transfected Hela Luc cells and MCF7 cells. Unlike the present HO-PBDEs, these HO-PCBs possessed tri- to tetrachlorination substitution on the phenolic ring. In this study, only three of the PBDEs (2,2',4,4',5-hexaBDE (BDE-153), 2,3,4,4',5,6-hexaBDE (BDE-166), and 2,3,3',4,4',5,6-heptaBDE (BDE-190)) showed antiestrogenic activities with concentrations resulting in 50% inhibition (IC50 values) ranging from 0.8 to 3.1 µM. These PBDEs are likely not metabolized in situ because the congeners are hexa- or heptabrominated, have two para-bromines, and have no adjacent or ortho-meta brominated carbons. Since the T47D Luc cells express a functional Ah receptor, it may be possible that the antiestrogenicity of these PBDEs is Ah receptor-mediated, as is the case for 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and several other antiestrogens (52). BDE-153, -166, and -190 induced the highest maximal luciferase activity in an Ah receptor CALUX assay based on H4IIE Luc cells, among the same set of 17 PBDEs (21).

The antiestrogenicity of Ah receptor ligands is directly correlated to their affinity for the Ah receptor and their CYP1A-inducing potency (52). As shown for TCDD-treated MCF7 cells (53), the result is enhanced estrogen catabolism, and lower availability of estrogen to the cell. This correlation between structure-antiestrogenicity- and structure-CYP1A-inducing potency has been shown for various halogenated aromatics such as TCDD and non-ortho PCBs in vivo and in vitro (55,56). The exact mechanism of antiestrogenicity is probably specific to species, cell type, and the estrogen-responsive gene. Other possible cellular mechanisms of Ah receptor-mediated antiestrogenicity of BDE-153, -166, and -190 may be that the Ah receptor decreases the binding of the ER to the estrogen-responsive element, or the Ah receptor could act as a repressor by inhibiting the binding of other transcription factors (ER) or the disruption of promoter function.

Interestingly, the HO-PBDEs induced luciferase to a higher maximum activity than the maximum induction generated by E2, though at higher concentrations. This has been shown for several other compounds mimicking the natural estrogen in reporter gene assays. Legler et al. (31) reported this phenomenon for the environmental estrogens genistein, nonylphenol, bisphenol A, o,p'-DDT, and methoxychlor in the same T47D Luc cells. Routledge and Sumpter (57) showed that genistein and 4-tert-octylphenol...
induced luciferase activity at a higher level than estradiol in a recombinant yeast strain. The mechanism of this high induction is not yet resolved, but effects on luciferase stability or stimulation of the expression of the receptor or co-activation factors are hypothesized to be involved (31).

We detected no striking differences in the relative binding affinities for the tested compounds between ERα or ERβ. However, the agonistic activity compared to E2 of BDE-30 pounds between ERα and BDE-100 was much higher in the 293-ERα-Luc cell line (Figure 6). Moreover, the agonistic activity of T2-like H-O-BDE, but not 4-phenoxy-phenol, was estrogen-receptor dependent (Figure 6). The induction of luciferase compared to E2 by T2-like H-O-BDE was much higher in the 293-ERα-Luc assay, whereas the induction of luciferase by 4-phenoxy-phenol was not detected by the assay. This would suggest that the presence of a bromine atom adjacent to the phenolic hydroxy group is a discriminating factor leading to a partial agonistic activity in the 293-ERα-Luc cell line compared to a full agonistic activity in the 293-ERα-Luc cell line.

In the same two ER-CALUX assays, polycyclic musk compounds were selective to the 293-ERα-Luc but not the 293-ERβ-Luc assay (32). H-O-PCBs with chlorine atoms only on the nonphenolic ring were found to bind with purified human ERα and ERβ with at least a 10-fold greater affinity than H-O-PCBs with chlorine atoms on the phenolic ring (34). However, the binding preference was 2-fold greater for the ERβ over the ERα. In the same study, 4-HO-13. Meerts IATM, Van Zanden JJ, Luijks EAC, Van Leeuwen-ronse et al. Environ Health Perspect 107(suppl 8):643–668 (1999).
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