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Structure-dependent activities of hydroxylated polybrominated diphenyl ethers on human estrogen receptor

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ABSTRACT

Polybrominated diphenyl ethers (PBDEs) have been shown to affect the estrogen receptor (ER) signaling pathway, and one of the proposed disruption mechanisms is direct binding of hydroxylated PBDE (OH-PBDE) to ER. In this paper, the binding affinity of 22 OH-PBDEs with different degrees of bromination to ER was assessed quantitatively using a surface plasmon resonance biosensor technique. Seven OH-PBDEs were found to bind directly with ER with K_D ranging from 1.46×10^{-7} M to 7.90×10^{-6} M, and the affinity is in the order of 6-OH-BDE-047 ≧ 4′-OH-BDE-049 > 4′-OH-BDE-017 > 6′-OH-BDE-099 ≧ 5′-OH-BDE-099 > 2′-OH-BDE-007 > 3′-OH-BDE-028. In MVLN luciferase gene reporter assays, 10 low-brominated OH-PBDEs induced luciferase activity alone, but are 10^5 to 10^7 fold less potent than E_2. Their estrogenic activity is in the order of 4′-OH-BDE-049 > 4′-OH-BDE-017 > 2′-OH-BDE-007 > 3′-OH-BDE-028 > 3-OH-BDE-047 ≧ 3′-OH-BDE-007. The good correlation between estrogenic activity and ER binding affinity of the low-brominated OH-PBDEs strongly suggest that these compounds induce ER transcriptional activity by binding directly with ER. The other 12 high-brominated OH-PBDEs inhibited luciferase activity of E_2 to various degrees, demonstrating their antagonistic activity. Molecular docking analysis of the ER/OH-PBDE complexes revealed two distinctive binding modes between low- and high-brominated OH-PBDEs which provided rationale for the difference in their ER activity.

1. Introduction

Over the past few decades, polybrominated diphenyl ethers (PBDEs) have been widely used as flame retardant additives in a variety of industrial and consumer products. As a result, elevated levels of PBDEs have been detected in environmental media and the bodies of human and wildlife (Hites, 2004; Mazdai et al., 2003; Meironyte et al., 1999). In 2004, the European Union began to prohibit the use of two PBDE commercial mixtures, penta-BDE and octa-BDE. Later in 2006, the US manufacturer stopped manufacturing these two PBDE mixtures. But due to their persistence in the environment and bioaccumlative nature in the food web, PBDE concentrations in the environment have been increasing (Schecter et al., 2005; She et al., 2002). High levels of both PBDEs and hydroxylated PBDEs (OH-PBDEs) have been found in human serum samples (Sjodin et al., 2004; Athanasiadou et al., 2007). The sources of OH-PBDEs are either the bio-transformation of PBDEs, or the natural products of some marine invertebrates (Wiseman et al., 2011).

To date, many studies have focused on PBDEs' toxic effects through some nuclear hormone receptor (NR) mediated pathways, in particular the pathways involving thyroid hormone receptor (TR), estrogen receptor (ER) and aryl hydrocarbon receptor (AhR) (Hamers et al., 2006; Kitamura et al., 2008; Wiseman et al., 2011). The estrogenic activity of PBDEs has been demonstrated in both experimental animals and cells. Several studies have shown that, when exposed to rats, BDE-99 disrupted the regulation of estrogen target genes (Ceccatelli et al., 2006), impaired spermatogenesis (Kuriyama et al., 2007), decreased circulating sex steroids and reduced anogenital distance in males (Lilienthal et al., 2006). Recent studies have shown that the neurotoxicity and endocrine disrupting effects of PBDEs might be enhanced by metabolizing to OH-PBDEs (Meerts et al., 2001; Hamers et al., 2006; Dingemans et al., 2008). As a result, OH-PBDEs have become the focus in toxicity studies.

The mechanisms of PBDE and OH-PBDE toxicity are complex and have not been fully resolved. In the only report on direct ER binding measurement, six OH-PBDEs (4′-OH-BDE-17, 2′-OH-BDE-28, 4-OH-BDE-42, 3-OH-BDE-47, 6-OH-BDE-47, 4′-OH-BDE-49) showed competitive binding with ERα (Mercado-Feliciano and Bigsby, 2008). In vitro estrogenic activity of OH-PBDEs has been investigated by cell transactivation assays in different cell lines. Two thyroid hormone-like OH-PBDEs were tested to be ER agonists by ER-CALUX assays (Hamers et al., 2006, 2008; Meerts et al., 2001). Using ER-CALUX assay in MCF-7 breast cancer

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cells, Kitamura et al. found that 3′-OH-BDE-7 and 4′-OH-BDE-17 exhibited estrogenic activity, but 4-OH-BDE-42, 3-OH-BDE-47, 4′-OH-BDE-49, and 4-OH-BDE-90 did not (Kitamura et al., 2008). Similarly, 4′-OH-BDE-17 and 4-OH-BDE-42 showed estrogenic activity, whereas 6-OH-BDE-47 antagonized the effect of E_2 at high concentrations (Mercado-Feliciano and Bigsby, 2008). In addition, 4′-OH-BDE-17 and 4-OH-BDE-42 showed agonistic activities in ER-CALUX assays in BG1Luc4E2 ovarian cancer cells, and 4′-OH-BDE-49 showed antagonistic activity (Kojima et al., 2009). Recently, Liu et al. studied the estrogenic potential of four OH-PBDEs by reporter gene assays. It was found that 4′-OH-BDE-17 showed strong estrogenic activity, whereas 2′-OH-BDE-28, 6-HO-BDE-47, and 4′-OH-BDE-49 exhibited anti-estrogenic potency (Liu et al., 2011).

Although the data are contradictory for some OH-PBDEs, the general trend seems to be that low-brominated OH-PBDEs act as ER agonists, whereas high-brominated ones are antagonists. However, due to the limited number of OH-PBDEs investigated in each study, the relationship between OH-PBDE structure and their estrogenic activity cannot be established convincingly. Furthermore, most of the studies employed transactivation reporter gene assays to characterize receptor-mediated estrogenic activity and elucidate the mechanisms of action without assessing the binding affinity of OH-PBDEs directly with ER. As suggested by Freyberger and Schmuck (Freyberger and Schmuck, 2005), some compounds could exhibit anti-estrogen-like activity without true binding affinity for ER. Therefore, it is advisable to investigate both direct ER binding affinity and ER-mediated transcription activity for the same compound.

In the present study, we employed a surface plasmon resonance (SPR) biosensor technique and measured the binding affinity of twenty-two OH-PBDEs with ER-LBD. These OH-PBDEs carry different numbers of bromines at different positions of the diphenyl rings. The SPR sensor technique can monitor the molecular interactions in real time, without labeling, and also provides kinetic parameters for ligand association and dissociation. By using the combination of SPR ligand binding assay, transactivation reporter gene assay and molecular docking, the estrogenic activity of these OH-PBDEs was investigated and analyzed.

2. Materials and methods

2.1. Materials

All the twenty two OH-PBDEs including 2′-hydroxy-4-monobromodiphenyl ether (2′-OH-BDE-003), 3′-hydroxy-2,4-dibromodiphenyl ether (3′-OH-BDE-007), 2′-hydroxy-2,4-dibromodiphenyl ether (2′-OH-BDE-007), 4′-hydroxy-2,2′,4-tribromodiphenyl (4′-OH-BDE-017), 3′-hydroxy-2,4,4′-tribromodiphenyl ether (3′-OH-BDE-028), 2′-hydroxy-2,4,4′-tribromodiphenyl ether (2′-OH-BDE-028), 4-hydroxy-2,2′,3,4′-tetrabromodiphenyl ether (4-OH-BDE-042), 4′-hydroxy-2,2′,4,5′-tetrabromodiphenyl ether (4′-OH-BDE-049), 3-hydroxy-2,2′,4,4′-tetrabromodiphenyl ether (3-OH-BDE-047), 5-hydroxy-2,2′,4,4′-tetrabromodiphenyl ether (5-OH-BDE-047), 6-hydroxy-2,2′,4,4′-tetrabromodiphenyl ether (6-OH-BDE-047), 2′-hydroxy-2,3′,4,5′-tetrabromodiphenyl ether (2′-OH-BDE-068), 4-hydroxy-2,2′,3,4′,5-pentabromodiphenyl ether (4-OH-BDE-090), 6-hydroxy-2,2′,3,4,4′-pentabromodiphenyl ether (6-OH-BDE-085), 6-hydroxy-2,2′,3,3′,4-pentabromodiphenyl ether (6-OH-BDE-082), 6′-hydroxy-2,2′,4,4′,5-pentabromodiphenyl ether (6′-OH-BDE-099), 5′-hydroxy-2,2′,4,4′,5-pentabromodiphenyl ether (5′-OH-BDE-099), 3-hydroxy-2,2′,4,4′,6-pentabromodiphenyl ether (3-OH-BDE-100), 6-hydroxy-2,3,3′,4,4′,5′-hexabromodiphenyl ether (6-OH-BDE-157), 6-hydroxy-2,2′,3,4,4′,6′-hexabromodiphenyl ether (6-OH-BDE-140), 3′-hydroxy-2,2′,4,4′,5,6′-hexabromodiphenyl ether (3′-OH-BDE-154), and 4-hydroxy-2,2′,3,4′,5,6,6′-heptabromodiphenyl ether (4-OH-BDE-188) were purchased from AccuStandard (New Haven, CT, USA) as solutions in acetonitrile with concentrations ranging from 10 μg/mL to 50 μg/mL. They were of 99% or higher purity. More concentrated stock solutions for our experiments were prepared by evaporating the solvent of the purchased solutions under a gentle stream of nitrogen and then dissolving the solid into 1 mM solutions in DMSO. 17β-Estradiol (E_2) and 4-hydroxytamoxifen (OHT) were bought from Sigma Aldrich (St. Louis, MO, USA). The chemical structures of these compounds are shown in Fig. 1. Testosterone was from Selleck Chemicals (Houston, TX, USA). N-(3-dimethylaminopropy)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), GST capture kit (with polyclonal anti-GST antibody, recombinant GST and glycine-HCl pH 2.1 as regeneration solution) and CM5 sensor chips were from GE Healthcare (Uppsala, Sweden). Recombinant human estrogen receptor alpha ligand binding domain (hERα-LBD) with GST-tag was from Invitrogen (Carlsbad, CA, USA).

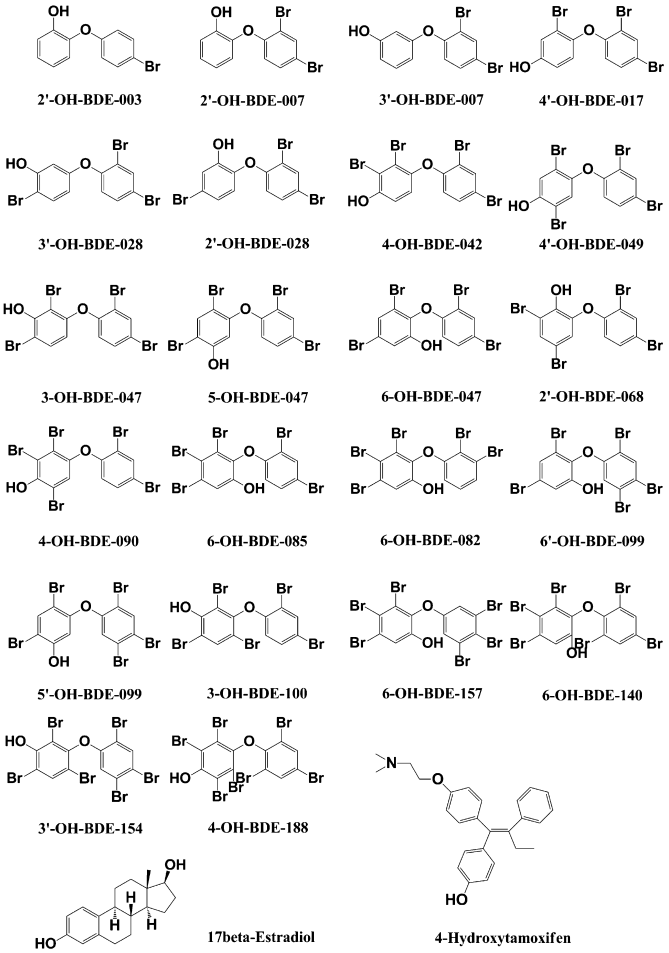

Fig. 1. Structures of the twenty-two hydroxylated polybrominated diphenyl ethers, estradiol, and 4-hydroxytamoxifen investigated in this study.

2.2. Measurement of OH-PBDEs binding with hERα-LBD

Direct binding of OH-PBDEs with hERα-LBD was measured by surface plasmon resonance (SPR) technique on a Biacore 3000 optical biosensor system (Biacore, Uppsala, Sweden). The instrument measures the SPR signal of the sensor in real time as a result of ligand binding to the receptor immobilized on the sensor surface and subsequent change of the refractive index at the sensor/liquid interface. The design of the sensor surface is illustrated in Figure S1.

Firstly, anti-GST antibody was immobilized on the CM5 sensor surface in both the sample cell and control cell, using the standard amine-coupling procedure and HBS-EP (0.01 M HEPES, 0.15 M NaCl, 0.005% v/v Surfactant P20, pH 7.4) as the running buffer (Karlsson et al., 2000). Forty microliters of a 30 μg/mL anti-GST antibody solution (in 10 mM sodium acetate, pH 5.0) was flew through the activated sensor surface for 4 min at 10 μL/min, followed by a 7 min injection of 1 M ethanolamine (pH 8.5) to block the remaining activated group on the surface. The high affinity sites of anti-GST antibody on the surface were blocked by running 2 cycles of 4-min injection of recombinant GST at a concentration of 5 μg/mL in HBS-EP buffer to make the surface regeneration possible. Secondly, GST tagged hERα-LBD (1.5 μM in 20 mM sodium phosphate, 150 mM sodium chloride, pH 7.4) was injected over the sample cell for 5 min. The receptor was captured by the anti-GST antibody on the sensor surface, resulting in an SPR signal of about 1800 RU.

Ligand binding measurement was carried out by passing a ligand solution through both the sample cell (with ER) and the control cell (without ER) at 30 μL/min, and recording the SPR signal as a function of time. Immediately before the measurement, OH-PBDEs stock solutions in DMSO were diluted into 20 mM sodium phosphate, 150 mM sodium chloride, pH 7.4 to yield 30 μM aqueous stock solutions (with 3% residual DMSO). Concentration series of a ligand were then prepared by serial dilution of the aqueous stock solution in the same buffer. The association and dissociation phase of the SPR measurement were 60 s and 180 s respectively for

each ligand. The kinetic constants were obtained by fitting the experimental data to a reversible 1:1 bimolecular interaction model included in the BIAevaluation software, Version 4.1 from the instrument manufacturer. After each ligand analysis, the receptor–ligand complex was stripped off the antibody-modified sensor surface by injecting 30 μL of regeneration buffer (glycine–HCl, pH 2.1) at 10 μL/min.

2.3. Cell transcription activity assay

MVLN cells for estrogen specific transcription assays were kindly provided by Prof. J.P. Giesy at Michigan State University, East Lansing, MI, USA. The assay utilizes an MCF-7 cell derivative that has been stably transfected with Vit-Luc reporter gene, and can be used to characterize estrogenic chemicals (Pons et al., 1990). Therefore, the estrogen specific transcription activity of a test chemical is directly related to the activity of luciferase in the lysate of treated MVLN cells.

MVLN cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM, Cellgro, Manassas, VA, USA) containing 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 100 U/mL streptomycin-penicillin (Gibco), 2 mM L-glutamine (Gibco) under humidified atmosphere of 5% CO_2 in a 37 °C incubator. For routine passages, the MVLN cell monolayer was removed with trypsin/EDTA (also from Cellgro) treatment, diluted to 10 mL with DMEM and cultured in 100-mm culture dishes.

The day after seeding in dishes, MVLN cells were changed into a steroid-free medium in order to increase the cell's hormonal sensitivity and decrease the background. The medium consisted of phenol red-free DMEM (from Cellgro) supplemented with 2% dextran-charcoal-treated fetal bovine serum (Hyclone, Logan, UT, USA), 100 U/mL streptomycin-penicillin, 2 mM L-glutamine, and was almost devoid of estrogens. Two days later, the cells were trypsinized with 0.25% trypsin (Cellgro) diluted in HBSS (Hank's Balanced Salt Solution, 8 g/L NaCl, 0.4 g/L KCl, 1 g/L glucose, 60 mg/L KH_2PO_4, 47.5 mg/L Na_2HPO_4, pH 7.2) without phenol-red, and seeded in a 96-well plate (Packard Instrument, Boston, MA, USA) at a density of 3×10^4 cells/well. After starving for 48 h, the cells were exposed to a test compound with or without E_2 for 48 h. Immediately before the exposure, the stock solution of each compound in DMSO was diluted into the steroid-free medium to yield a 10 μM solution (with 0.1% residual DMSO). A concentration range from 4.6 nM to 3.3 μM for each compound was then prepared by serial dilution of the 10 μM stock solution with the steroid-free medium containing 0.1% DMSO. At the end of chemical exposure, luciferase activity of the cell was measured using the Luciferase Assay System (Progema, Madison, WI, USA) according to the manufacturer's protocol. Luminescence signal was measured on a Varioskan Flash microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) with a 10 s integration time.

Cell viability was determined in parallel with MVLN assay using the WST-1 proliferation kit (Roche Diagnostics, Mannheim, Germany). Briefly, at the end of treatment by the tested compound, 200 μL steroid-free medium and 20 μL WST-1 were added into each well of the 96-well plate and incubated for 4 h at 37 °C in humidified 5% CO_2. The absorbance of the cells in each well was measured at 450 nm using the Varioskan Flash microplate reader.

2.4. Data analysis

In the cell assays, each concentration of a chemical was tested in triplicate wells, and each chemical was tested in two dependent experiments. All the results are expressed as the mean ± SD. We used the one-way analysis of variance (ANOVA) and *t*-test to assess the significance of the difference. Difference was considered significant at a *p*-value ≤0.05.

The estrogenic effects of OH-PBDEs were calculated according to Eq. (1),

$$E(\%) = 100 \times \left(\frac{L_{test} - L_{control}}{L_{E_2} - L_{control}} \right) \quad (1)$$

where E is the percent of estrogenic effects, and L_{test}, $L_{control}$, and L_{E_2} are the average luciferase activity of test compounds, solvent control (0.1%), and 1 nM E_2 respectively.

The concentration–response analyses were performed with four-parameter logistic curve regression analysis according to the following equation:

$$y = \frac{\text{minimum} + (\text{maximum} - \text{minimum})}{[1 + (x/\text{EC}_{50})^{Hill\ slope}]} \quad (2)$$

where y is the response value, x is the log concentration of the test compound, and EC_{50} is the concentration that induces half the maximum proliferation effect or luciferase activity. EC_{50} values were calculated as

$$EC_X = \left[\frac{x}{(100 - x)} \right]^{(1/Hill\ slope)} \times \text{EC}_{50} \quad (3)$$

where x is 20% of the maximum effects, and the Hill slope and EC_{50} were calculated from Equation 2.

All statistical analyses were performed using Origin (version 8.0; OriginLab Inc., Northampton, MA, USA).

2.5. Molecular docking

The crystal structure of hERα-LBD/OHT complex was obtained from the Protein Data Bank (PDB ID 3ERT) (Shiau et al., 1998). E_2, OHT and OH-PBDEs were docked to hERα-LBD using Lamarckian genetic algorithm provided by AutoDock 4.2 software (Morris et al., 2009). Grid boxes were centered at the core of ER-LBD binding pocket and built around the protein with 60 points cube coverage. A spacing of 0.375 Å between the grid points was used. All other docking parameters were set to defaults, including a population size of 150, maximum number of 2.5 million evaluations, maximum of 2700 generations, gene mutation rate of 0.02, crossover rate of 0.8, and 10 GA runs. Ten docked conformations for each ligand were scored according to a free energy cost function (ΔG^*), and the first ranked conformation was selected for analysis.

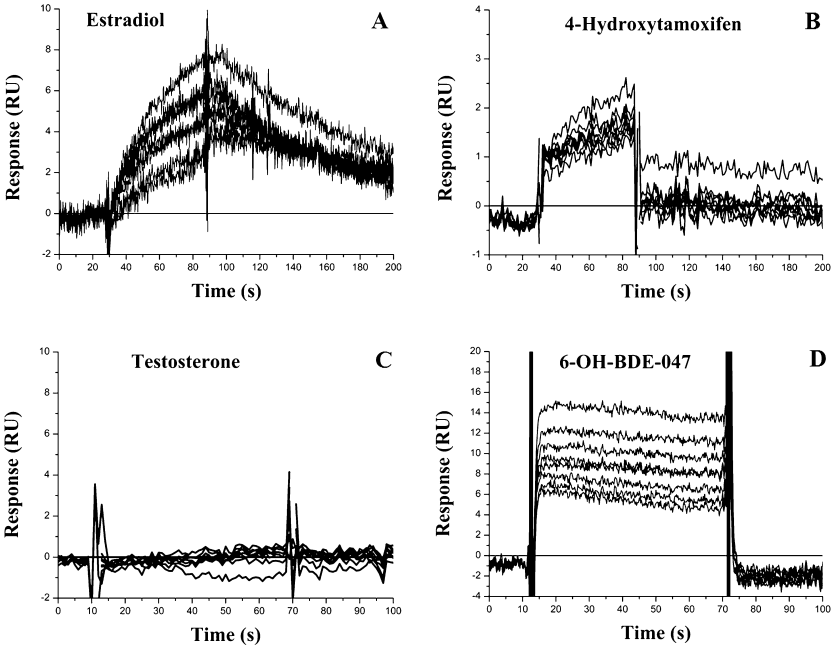

Fig. 2. SPR response curves of (A) E_2, (B) OHT, (C) testosterone, (D) 6-OH-BDE-047 binding with ER-LBD immobilized on the sensor chip. A ligand was injected at a concentration of 1.5, 4.6, 14, 41, 124, 370, 1111 or 3333 nM respectively for 60 s, followed by a dissociation phase of 180 s.

Table 1
Rate constants and relative binding potency values obtained in the SPR measurement of ligand binding with ER-LBD.

Ligand	Bromine Substitution	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (M)	Relative Binding Potency (%)
E_2	–	1.91E+7	6.64E-3	3.47E-10	100.00
OHT	–	1.75E+3	1.00E-5	5.73E-9	6.06
2′-OH-BDE-007	2,4	3.36	1.00E-5	2.98E-6	0.012
4′-OH-BDE-017	2,2′,4	13.50	1.00E-5	7.39E-7	0.047
3′-OH-BDE-028	2,4,4′	8.48	6.70E-5	7.90E-6	0.004
4′-OH-BDE-049	2,2′4,5′	59.2	1.00E-5	1.69E-7	0.21
6-OH-BDE-047	2,2′,4,4′	68.5	1.00E-5	1.46E-7	0.24
6′-OH-BDE-099	2,2′4,4′,5	7.04	9.93E-6	1.41E-6	0.025
5′-OH-BDE-099	2,2′4,4′,5	5.87	1.00E-5	1.70E-6	0.020

3. Results and discussion

Binding to ER is the first necessary step in ER mediated transcriptional activities of a ligand. The binding potency of 22 selected OH-PBDEs with ER-LBD was assessed quantitatively by SPR technique, using E_2 (an endogenous estrogen) as the positive control and reference. Shown in Fig. 2A is the SPR response curve of E_2 as a function of time. As E_2 was passed over the ER-LBD modified sensor surface, SPR signal increased due to E_2 binding. After this 60 s association phase, the solution was changed to the blank, and the SPR signal decreased over time (the 180 s dissociation phase). From the kinetic curve, binding parameters such as k_a (association rate constant), k_d (dissociation rate constant), and K_D (equilibrium dissociation constant) were calculated. The K_D value of E_2 obtained in the SPR measurement (0.35 nM) is in good agreement with the results of fluorescence polarization (0.60 nM) (Bolger et al., 1998), radio-labeled competitive assay (0.26 nM) (Gangloff et al., 2001) and other SPR based assay (0.90 nM) (Rich et al., 2002). Similarly, the K_D value of OHT (6.9 nM), a known ER antagonist, falls into the range of 0.2–18 nM measured by other methods (Labrie et al., 1999; Rich et al., 2002; Sun et al., 1998). Testosterone is a hormone that has no effect on ER. As expected, its SPR response remained close to the baseline (Fig. 1C), demonstrating the selectivity of this method.

After validating the SPR method, the binding potency of 22 OH-PBDEs with ER-LBD was then investigated. These chemicals are diversely structured, with the bromine number ranging from one to seven, and also with different bromine positions. Seven out of the twenty-two OH-PBDEs showed direct binding reaction to ER-LBD, as demonstrated in Fig. 2D and Figure S2, but the other 15 compounds did not. The K_D values of the seven OH-PBDEs are in the range of 1.46×10^{-7} M to 7.90×10^{-6} M, and the binding affinity is in the order of 6-OH-BDE-047 ≧ 4′-OH-BDE-049 > 4′-OH-BDE-017 > 6′-OH-BDE-099 ≧ 5′-OH-BDE-099 > 2′-OH-BDE-007 > 3′-OH-BDE-028. Compared to E_2, these OH-PBDEs have significantly low binding affinity with ER-LBD. Even the most potent one, 6-OH-BDE-047, has a relative binding potency of 0.24%. All the binding parameters and relative binding potency values are listed in Table 1. In a previous report on ER binding measurement, six OH-PBDE metabolites (4′-OH-BDE-17, 2′-OH-BDE-28, 4-OH-BDE-42, 3-OH-BDE-47, 6-OH-BDE-47, 4′-OH-BDE-49) displaced ^{3}H-estradiol from recombinant ERα (Mercado-Feliciano and Bigsby, 2008). The relative binding affinity for 4′-OH-BDE-17, 6-OH-BDE-47 and 4′-OH-BDE-49 is different from our results, possibly due to the use of different proteins and measurement methods.

MVLN cells are known for their responsiveness to estrogens, and have been widely used to study ER activities of suspected estrogenic compounds (Freyberger and Schmuck, 2005). In the present study, we utilized a stably transfected MVLN cell line to investigate the estrogenic and anti-estrogenic effects of OH-PBDEs. Before the activity assay, cytotoxicity of each OH-PBDE was tested using the WST-1 assay to determine the suitable concentration range. As shown in Figure S3, for most of the 22 OH-PBDEs there was no significant effect on cell viability in the concentration range of 4.6 nM–10 μM (solubility limit). 3′-OH-BDE-154 and 4-OH-BDE-188 showed slight cytotoxicity at 10 μM, and this concentration was excluded in the data analysis.

All the OH-PBDEs were then tested for their estrogenic effects by treating with MVLN cells and measuring luciferase activity, using E_2 as the positive control. E_2 induced luciferase activity at concentrations above 10^{-13} M, and reached maximum activity at approximately 1 nM, with an EC_{50} of 12 pM (Fig. 3). This is in good agreement with published results (Wang et al., 2012). Ten of the twenty-two OH-PBDEs exhibited luciferase induction to various degrees (Fig. 3), indicating that these compounds are ER agonists. All the ten compounds induced luciferase activity at micromolar concentrations, which are 10^5 to 10^7 fold less potent than E_2. The calculated relative activity (EC_{20} of E_2 divided by that of an OH-PBDE) is listed in Table 2, and is in the order of 4′-OH-BDE-049 > 4′-OH-BDE-017 > 2′-OH-BDE-007 > 3′-OH-BDE-028 > 3-OH-BDE-047 ≧ 3′-OH-BDE-007. It is

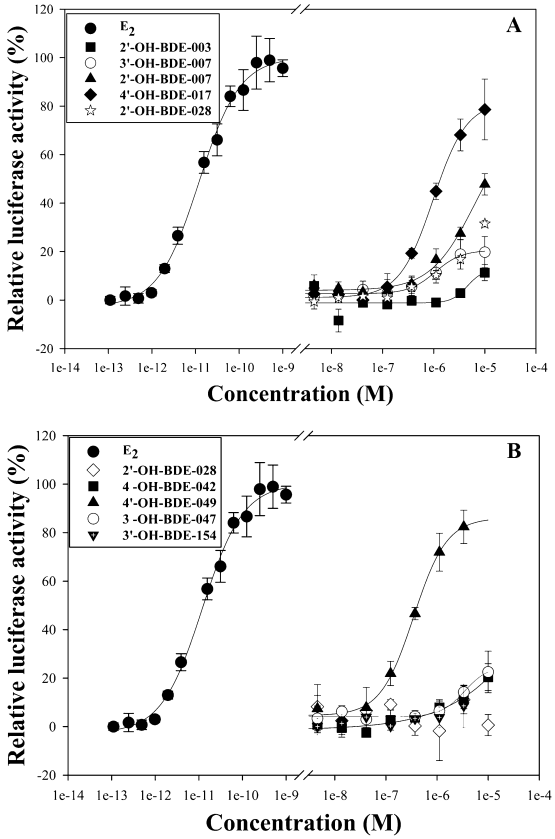

Fig. 3. Luciferase activity induced by OH-PBDEs alone in MVLN cells relative to the maximum induction by E_2 calculated by Eq. (1). The OH-PBDEs were tested at concentrations of 4.6, 13.7, 41.2, 123, 370, 1111, 3333, and 10,000 nM. E_2 concentrations ranged from 2.4×10^{-13} M to 1×10^{-9} M.

Table 2
Estrogenic activity of OH-PBDEs measured in MVLN cells as depicted in Fig. 3.

Ligand	Bromine Substitution	EC_{20} (M)[a]	Relative Activity (%)[b]
E_2	–	3.44E-12	100
2′-OH-BDE-003	4	NA[c]	–
3′-OH-BDE-007	2,4	7.06E-6	4.9E-5
2′-OH-BDE-007	2,4	2.20E-6	1.6E-4
4′-OH-BDE-017	2,2′,4	4.00E-7	8.6E-4
3′-OH-BDE-028	2,4,4′	4.10E-6	8.4E-5
2′-OH-BDE-028	2,4,4′	NA	–
4-OH-BDE-042	2,2′3,4′	NA	–
4′-OH-BDE-049	2,2′4,5′	1.08E-7	3.2E-3
3-OH-BDE-047	2,2′4,4′	6.53E-6	5.3E-5
3′-OH-BDE-154	2,2′,4,4′,5,6′	NA	–

[a] Concentration at which the induction of luciferase activity is 20% of the maximum induction by E_2 calculated by Eq. (3).
[b] Relative activity was calculated by dividing the EC_{20} of E_2 by that of an OH-PBDE.
[c] not available because the effect was insufficient to calculate an EC_{20}.

Table 3
Anti-estrogenic activity of OH-PBDEs measured in MVLN cells in the presence of 15pM E2, as depicted in Fig. 4.

Ligand	Bromine substitution	IC_{50} (M)[a]
OHT	–	1.09E-07
6-OH-BDE-047	2,2′,4,4′	2.25E-06
2′-OH-BDE-068	2,3′4,5′	6.02E-06
6-OH-BDE-082	2,2′,3,3′,4	2.27E-06
6-OH-BDE-085	2,2′,3,4,4′	1.02E-06
4-OH-BDE-090	2,2′,3,4′,5	5.47E-06
6′-OH-BDE-099	2,2′4,4′,5	1.99E-06
5′-OH-BDE-099	2,2′4,4′,5	6.34E-06
3-OH-BDE-100	2,2′,4,4′,6	6.67E-06
6-OH-BDE-140	2,2′,3,4,4′,6′	2.48E-06
6-OH-BDE-157	2,3,3′,4,4′,5′	6.67E-06
4-OH-BDE-188	2,2′,3,4′,5,6,6′	1.63E-06

[a] Concentration at which the induction of luciferase activity by 15pM E2 is inhibited by 50%.

worth noting that all these OH-PBDEs have 4 or less bromine atoms. The induction by 2′-OH-BDE-003, 2′-OH-BDE-028, 4-OH-BDE-042 and 3′-OH-BDE-154 was below 20%. Interestingly, there is a good correlation between the agonistic activity of an OH-PBDE in MVLN cells and its binding potency with ER-LBD measured by SPR. This may suggest that the ER activity of an OH-PBDE agonist is dictated by its direct binding affinity with ER.

To further investigate the estrogenic and anti-estrogenic effect of OH-PBDEs, MVLN cells were co-exposed to 15 pM E_2 (its EC_{50}) and various concentrations of an OH-PBDE. At 10 nM concentration, ER antagonist OHT completely inhibited the luciferase activity induced by 15 pM E_2, as expected. For the 22 OH-PBDEs, three distinctive effects were observed. Six OH-PBDEs (2′-OH-BDE-007, 4′-OH-BDE-017, 3′-OH-BDE-028, 4-OH-BDE-042, 3-OH-BDE-047 and 4′-OH-BDE-049) further increased luciferase activity in addition to E_2 (Fig. 4A). These low-brominated compounds, with the exception of 4-OH-BDE-042, showed agonistic activity when exposed alone (Fig. 3). Therefore, additional activity in the co-exposure with E_2 would be expected. Four other OH-PBDEs (2′-OH-BDE-003, 3′-OH-BDE-007, 2′-OH-BDE-028,

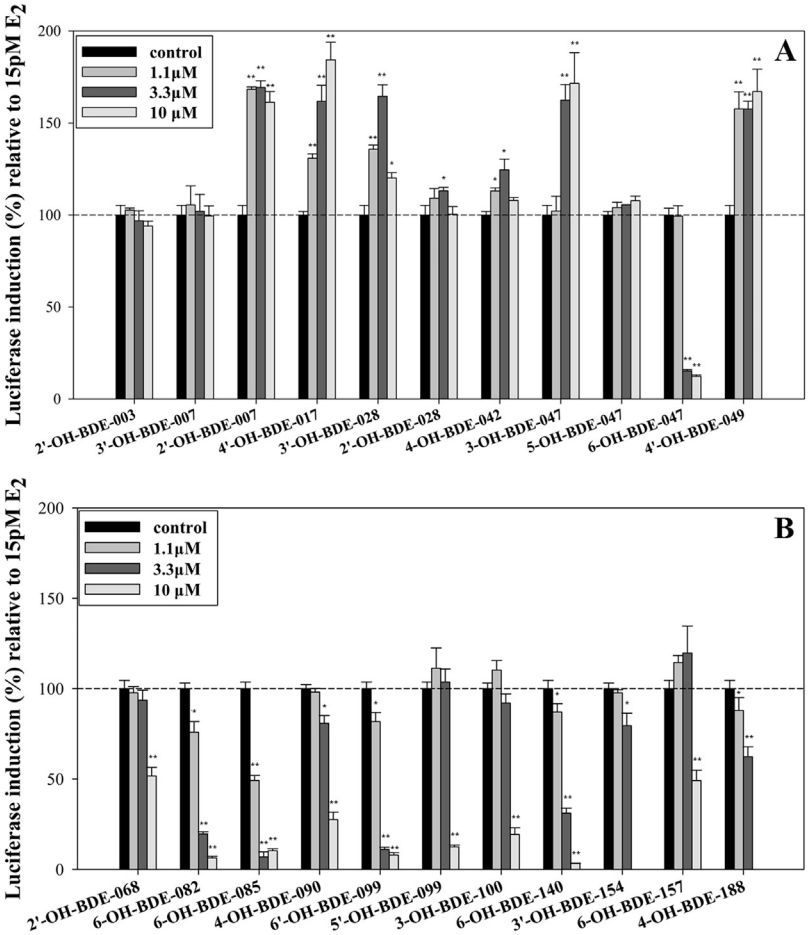

Fig. 4. Luciferase activity induced by OH-PBDEs in MVLN cells in the presence of 15 pM E_2. The activity is given relative to the induction by 15 pM E_2. The OH-PBDEs were tested at concentrations of 1.1 μM, 3.3 μM, and 10 μM. The error bar represents the standard deviation of three independent measurements. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with blank (no ligand).

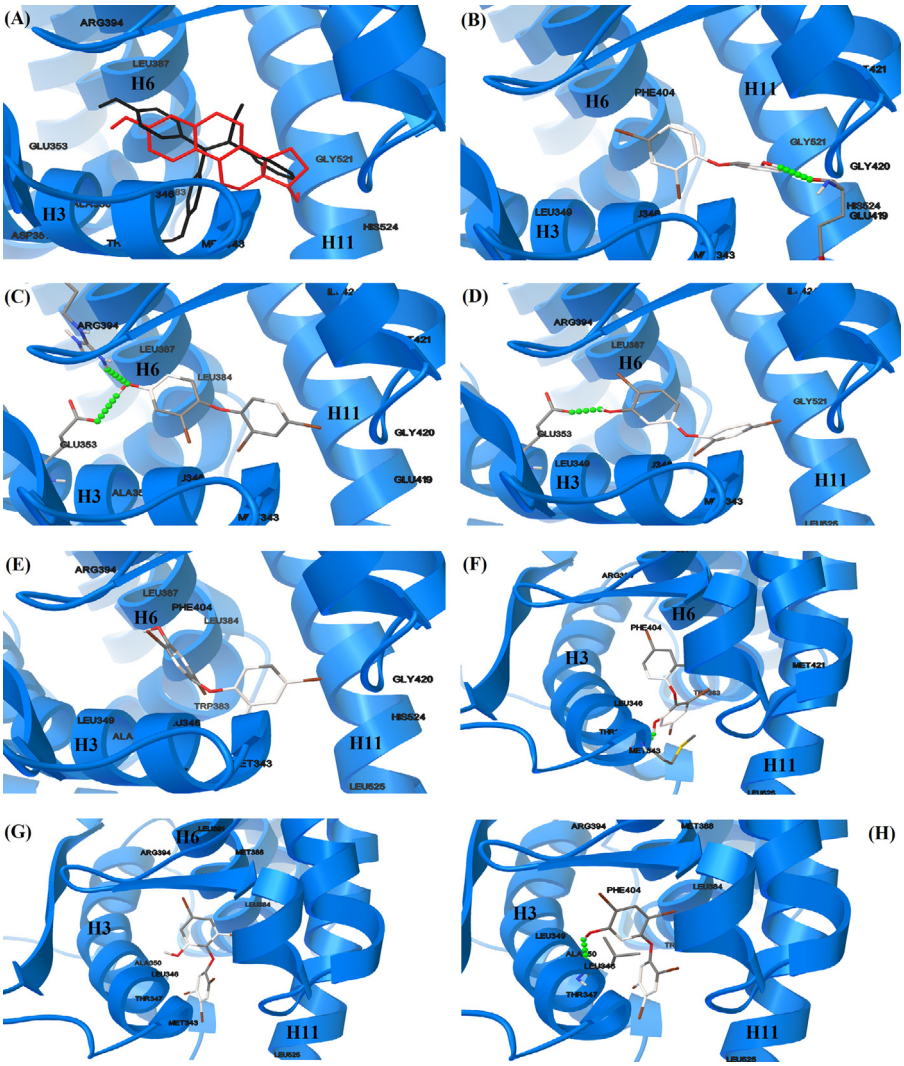

Fig. 5. Graphs for the docked complexes between hERα ligand binding domain and selected ligands. (A) pose comparison between E_2 (red) and OHT (black), (B) 2′-OH-BDE-007, (C) 4′-OH-BDE-017, (D) 3′-OH-BDE-028, (E) 4′-OH-BDE-049, (F) 6-OH-BDE-047, (G) 6′-OH-BDE-099, (H) 5′-OH-BDE-099. The structure with the lowest binding energy for each ligand is shown. The ligands and relevant amino acid residues of the receptor are colored by atom type (carbon in gray, nitrogen in blue, oxygen in red). Hydrogen bonds are represented by dotted green lines between the donor and acceptor.

5-OH-BDE-047) did not increase the activity up to 10 μM (Fig. 4A). Since they exhibited very low activity or no activity at all in the single exposure experiments, the lack of activity increase in the co-exposure experiments would also be expected.

For the remaining 12 OH-PBDEs, co-exposure with E_2 inhibited luciferase activity to various degrees (Fig. 4), demonstrating their antagonistic activity. Their IC_{50} values are in the micromolar range (Table 3), showing much less antagonistic potency than OHT. All these OH-PBDE antagonists possess four or more bromine atoms. Some of them, 6-OH-BDE-047, 5′-OH-BDE-099 and 6′-OH-BDE-099, were found in SPR measurement to interact directly with ER-LBD. However, the other nine OH-PBDE antagonists did not exhibit any ER binding capability. We suspect that these OH-PBDEs inhibit ER transcription activity by disrupting other interactions in the transcriptional process, which include ER dimerization, ER/DNA binding, and coactivator recruitment. In a recent report, Ibhazehiebo et al. demonstrated that some PBDE congeners disrupted the thyroid hormone receptor (TR) pathway by partially dissociating TR from the hormone response element (Ibhazehiebo et al., 2011). Similar disruption by OH-PBDEs may occur to the ER/DNA complex.

The in vitro estrogenicity of OH-PBDEs has been investigated previously by ERE-luc and ER-CALUX reporter gene assays in various cell lines (Hamers et al., 2006, 2008; Kojima et al., 2009; Liu et al., 2011; Meerts et al., 2001; Mercado-Feliciano and Bigsby, 2008). Although the data are sometimes inconsistent, it seems that in most assays low-brominated OH-PBDEs are ER agonists, and high-brominated ones are antagonists. Our results follow the same trend.

To understand the structural basis for the different ER activity between low- and high-brominated OH-PBDEs, the binding interaction of OH-PBDEs with ER-LBD was modeled by molecular docking. E_2 and OHT were first docked to ER-LBD using the Autodock software. The calculations were performed with both rigid and flexible amino acid side chains of the ER binding pocket. We found that the docking results were much more consistent with their crystallographic structures when the calculations were performed with rigid side chains. Some key different features were evident between the two docked complexes (Fig. 5A). Three hydrogen bonds were formed between the hydroxyl groups of E_2 and the side chain residues of Glu353 (H3), Arg394 (H6) and His524 (H11). E_2 was completely inside the binding pocket. For OHT, only two hydrogen bonds were found between its phenolic hydroxyl group and Glu353 and Arg394. Also, the long and bulky side chain of OHT protruded from the pocket between H3 and H11. This extension is believed

Table 4
Molecular docking results of the interactions between the selected ligands and hERα ligand binding domain.

Ligand	Bromine Substitution	Critical Volume (cm^3/mol)[a]	Hydrogen Bonds	ER Activity
E_2	–	821.5	ARG394, GLU353, HIS 524	agonist
OHT	–	1201.5	ARG394, GLU353	antagonist
2′-OH-BDE-007	2,4	649.5	GLU419	agonist
4′-OH-BDE-017	2,2′,4	711.5	ARG394, GLU353	agonist
3′-OH-BDE-028	2,4,4′	711.5	GLU353	agonist
4′-OH-BDE-049	2,2′,4,5′	773.5	–	agonist
6-OH-BDE-047	2,2′,4,4′	773.5	–	antagonist
6′-OH-BDE-099	2,2′,4,4′,5	835.5	–	antagonist
5′-OH-BDE-099	2,2′,4,4′,5	835.5	LEU346	antagonist

[a] Critical Volumes were calculated by ChemiBioDraw.

to prevent the fold-back of ER H12 which would seal the binding pocket and generate a hydrophobic groove for ER co-activators (Shiau et al., 1998). These modeling results are in good agreement with the crystallographic data (Shiau et al., 1998), even though water molecules such as the one between Arg394 and Glu353 in the crystal structure were deleted in the docking calculation.

After validating the docking method, the seven OH-PBDEs that showed direct binding with ER in the SPR measurement were docked with the receptor using the same parameters as E_2 and OHT. All the docked complexes are illustrated in Fig. 5. It is obvious that there exist two distinctive binding modes among the seven OH-PBDEs. Four of them (2′-OH-BDE-007, 4′-OH-BDE-017, 3′-OH-BDE-028, and 4′-OH-BDE-049) fit into the ER binding pocket completely, similar to E_2. However, the other three compounds (6-OH-BDE-047, 6′-OH-BDE-099, and 5′-OH-BDE-099) were docked to ER in a conformation that resembles OHT, with part of the molecule protruding into the space between H3 and H11 of ER. The reason for the different binding modes perhaps lies in the size of these compounds relative to the E_2 binding pocket of the receptor. As listed in Table 4, the critical volume of OHT (1201.5 cm^3/mol) is much larger than that of E_2 (821.5), and thus cannot be confined inside the binding pocket without strong torsion. This is why its side chain protrudes the binding pocket in the structure. The volume of low-brominated OH-PBDEs (1–4 bromines) is 773.5 or less, which is smaller than E_2. However, the volume of high-brominated OH-PBDEs (4 bromines or more) is much larger.

4. Conclusions

In conclusion, the binding interactions of 22 OH-PBDEs with human ER-LBD were investigated by surface plasmon resonance technique. The data provided direct evidence for the estrogenic/anti-estrogenic effect of seven OH-PBDEs acting through the interactions with ER-LBD. In ER transcription cell assays, low-brominated OH-PBDEs acted as ER agonists, whereas high-brominated compounds exhibited antagonistic activity. The potency of estrogenic OH-PBDEs was found to correlate well with their ER binding affinity, with 4′-OH-BDE-049 possessing the strongest activity. The difference in ER activity between low- and high-brominated OH-PBDEs was rationalized by molecular docking analysis with ER-LBD which revealed two distinctive binding modes depending on the molecular size of the ligand. Low-brominated OH-PBDEs were docked efficiently inside the E_2 binding pocket on ER, but high-brominated compounds extended beyond the pocket. Among the 22 OH-PBDEs we investigated, some (4′-OH-BDE-017, 2′-OH-BDE-028, 4-OH-BDE-042, 6-OH-BDE-047, 3-OH-BDE-047, 4′-OH-BDE-049, 4-OH-BDE-090, 5′-OH-BDE-099, 6′-OH-BDE-099) were found to be present in human blood samples (Athanasiadou et al., 2007; Qiu et al., 2009). Although the activities of these OH-PBDEs are not as strong as E_2 or OHT, due to the possibility of bio-accumulation and bio-amplification, their potential toxicity at elevated concentrations is still a matter of concern.

Conflict of interest

There are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tox.2013.04.001.

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