Estrogenic, Genotoxic and Mutagenic Toxicity Study of PBDEs

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1. Introduction

Polybrominated diphenyl ethers (PBDEs) are widely used as flame retardants in plastics, electronic appliances, vehicles, textiles and packaging materials[1]. There are 209 congeners and the commercial PBDEs are actually the mixtures of several different congeners with the three major products called Penta-BDEs, Octa-BDEs and Deca-BDEs[2]. The structural similarities of certain PBDEs congeners to other polyhalogenated aromatic hydrocarbons, such as polychlorinated biphenyls (PCBs) and Dioxins have raised concerns that PBDEs might activate similar toxic effects. Earlier works suggested that PBDEs may be considered to perform a similar action of PCBs interfering with the estrogenic pathway and inducing CYP 1A1/2 [3]. There was found that both commercial mixtures and individual congener had aryl hydrocarbon receptor (AhR) binding affinities 10^{-2} to 10^{-5} times that of TCDD using human, rat, chicken, and rainbow trout cells, however, lower brominated PBDEs congeners acted as AhR agonists and antagonists when co-treated with TCDD[4]. Some studies also indicated that does not induced AhR gene expression *in vivo* and *in vitro*[5,6]. Such as penta-BDEs were found to have a low affinity for the Ah receptor but were unable to induce dioxin response element binding[7]. Recent study indicated hydroxylated (OH-) PBDEs metabolites could interact with the thyroid hormone system and display agonistic or antagonistic activity toward the AhR[8] or induce the expression of CYP1A1/A2[9]. It is necessary to investigate whether PBDEs could induce the Ah receptor in vitro and in vivo to understand the potential of PBDEs effect on biota and humans.

Adequate data on the effects are currently still insufficient to fully understand their toxicology, especially for carcinogenicity, genotoxicity and mutagenicity, mechanism and pathway. It is proposed that the genotoxicity profiles of PBDEs and PCBs are similar [10]. However, based on in vitro assays it was found that BDE-209 did not induce gene mutations in bacteria (*S. typhimurium*), mammalian cells (mouse lymphoma L5178Y cells), and sister chromatid exchange or chromosomal aberrations in Chinese hamster ovary cells. The commercial mixtures did not impose mutagenicity on *S. typhimurium*. Mono- and di-BDEs induced genetic recombination in two mammalian cell lines (HeLaTR and MCF7) but the tetra-BDEs mixture was negative[11]. Lower brominated congeners tested positive in mutagenicity tests (such as the Ames test and in vitro tests of chromosomal aberrations)[12].

There is also a lack of data on carcinogenicity studies. Low brominated congeners other than BDE–209 would be more of a concern with respect to carcinogenicity since they would be more readily absorbed and more slowly eliminated[12]. Exposure of Chinese hamster cells to low brominated congeners caused increased gene recombination at the HGPRT locus (Helleday et al., 1999). In addition, such toxicological data related to PBDEs are limited and are primarily obtained by studying commercial mixtures [13].

In the present investigation, the estrogenic and genotoxic effects of lower and higher brominated congeners are studied using several bio-assays. The estrogenic effects will be investigated by recombinant yeast cell exposed to PBDEs, dioxin-like activities by EROD assays and genotoxicity/mutagenicity by SOS/*umu* assays.

2. Materials and Methods

2.1 Chemicals

All PBDEs standard solutions were purchased from Wellington Laboratories (Ontario, Canada): eight standard solutions containing 7 native PBDEs: BDE-28, -47, -99, -153, -154, -183, and -209 (5 ng/L in nonane), and commercial product DE–71 (penta-BDEs including di- to hexa-BDEs) and Deca-BDE (solid). PBDEs spiked in dimethyl dulfoxide (DMSO) ranged from 0.1 to 50 μM. 2, 3, 7, 8-TCDD (>99% pure) were purchased from Cambridge Isotope Laboratories (Woburn, MA, USA). Dulbecco's modified eagle medium (Dulbecco's MEM) containing D-Glucose (1.0 g/L) and n-acetyl-l-alanyl-l-glutamine (3.7 g/L) was purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany). Bactotryptone, bovine serum, penicillin, streptomycin, cupric sulfate (CuSO4), 4-nitroquinoline-N-oxide (4-NQO), 7-ethoxyresorufin, dicumarol, bactotryptone, sodium chloride (NaCl), and glucose were purchased from Sigma (Deisenhofen, Germany). Ortho-nitrophenyl-b-D-galacto -pyranoside (ONPG) was dissolved in dimethylsulfoxide (DMSO) (cell culture grade) (Tedia Company Inc. Fairfield, OH, USA).

2.2 Recombinant Yeast Assay

The recombinant yeast strain (*Saccharomyces cerevisiae*) was provided by Dr. J. Sumpter in Brunel University, UK. Yeast culture and galactosidase measurements were carried out according to the procedures described by Routledge and Sumpter with slight modifications by Wu et al. Briefly, 50–100 μL of a concentrated recombinant yeast stock was added to 10 mL SC medium with 10 μL CuSO4 and incubated at 30 °C for 20 h in a shaking incubator (130 r/min) (Medtrue enterprise Co., Ltd., China), then the assay medium was diluted with SC medium until an optical density (OD) of 0.75 measured at a wavelength of 600 nm with a spectrophotometer was reached. PBDEs standard solutions (ranging from 0.1 to 50 μM) were prepared. The dilution series, with DMSO solvent blank as the negative control and the 17***β***-estradiol (E_2) as the positive control, were tested in triplicate. Clear plastic 96-well plates were seeded with 200 μL per well of the assay medium containing yeast and dilution series. A calibration curve was performed by E_2 standard gradient (0.01-20 nmol/mL in DMSO, N=11). Measurements of luciferase activity and calculation of estradiol equivalent concentrations (EEQs) of samples by interpolation in standard curves were performed as described using E2 as a positive control.

2.3 Ethoxyresorufin-O-Deethylation Assays

In vitro: Rat hepatoma cell lines (H4IIE) were obtained from the American Type Culture Collection, and cultured with the cell culture medium containing Dulbecco's MEM, 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. The cell culture and EROD bioassay were performed following Donato's method. There were three duplicates for each concentration (0.1-10 μM) and solvent blank had on one 96-well plate. H4IIE cells were exposed to increasing concentrations of PBDEs (0.1-10 μM), the positive control TCDD (0.01–2.5 nM), and the negative control DMSO (0.1%). After 72 h, the media were removed and 100 μL 5 mM 7-ethoxyresorufin and 0.1 μL 10 mM dicumarol were added to each well. The conversion of ethoxyresorufin to resorufin was measured under an excitation wavelength of 530 nm and an emission wavelength of 590 nm using HTS 7000 bioassay reader.

In vivo: Fish liver cells were collected from Japanese medaka (*Oryzias latipes*) exposed to BDE–209 in the treated groups ranging from 1.0 ng/L to 100 μg/L after one, two and three weeks under the flow-through exposure system (see Chapter VI for detail). A The TCDD equivalent concentrations (TEQs) in PBDEs were then calculated according to the calibration curve. The minimum detectable concentration was 0.02 pg TEQ/well.

2.4 SOS/*umu* Assays

Salmonella typhimurium TA1535/pSK1002, whose plasmid pSK1002 carries an u*mu*C/*lac*Z fused gene, was provided in 2006 by Osaka Prefectural Institute of Public Hygiene, Japan. The culture was diluted fold with therapeutic goods administration (TGA) medium consisting of 1% bactotryptone (w/v), 0.5% NaCl (w/v) and 0.2% glucose (w/v), and further incubated at 37 ℃ for 1 h until the bacterial OD600 reached about 0.5 (from 0.2 to 0.6) measured by a Fluostar fluorescence plate reader (BMG). Each sample was assayed using duplicate tubes. Clear plastic 96-well plates were seeded with 200 μL per well of the assay medium containing yeast and incubated at 37 ℃ for 2 h. The dilution culture (990 μL) was added to the pipes, followed by the addition of 10 μL PBDEs congeners (0.1-10 μM) as well as negative (DMSO) and positive (4-NQO) (50–300 μg/mL) controls dissolved in DMSO. For testing the co-exposure of bacterial cells to PBDEs and 4-NQO, the diluted culture (980 μL) was added with to 10 μL of PBDEs congeners (0.1–10 μM), together with 10 μL (4-NQO) (50-300 μg/mL) at a series of concentrations respectively. The cytotoxic effects were determined in the reaction mixtures by measuring the absorbance at 600nm of each microplate well, to quantify growth inhibition in BMG. The expressed ***β***-galactosidase activity in DMSO, the tester strain, was determined using 4-nitroquinoline-N-oxide (4-NQO) as a substrate. These mixtures were incubated at 37 ℃ for 1 h with vigorous shaking, and then the bacterial density and the ***β***-galactosidase activity were measured at the optical density (420 nm and 550 nm). The result was considered as positive if the IR was higher than 2.0, which meant that the genotoxic potency of the sample to induce a two-fold increase to that of negative.

3 Results and Discussion

The responses of recombinant yeast cell exposed to the 7 most common congeners of PBDEs (BDE-28, -47, -99, -153, -154, -183, and -209) and the commercial product DE-71 are summarized and results indicate that individual congeners did not significantly ($p>0.05$) induce ***β***-galactosidase at exposure concentrations from 1 to 50 μM. Weak estrogenic effects were observed for BDE-153, -28, -47, -209, -183 and DE–71 at exposure concentration 1 μM. Antiestrogenic activity of PBDEs have also observed when T47D. Luc cell (breast carcinoma cell lines) was treated in combination with 17***β***-estradiol (E_2) and BDE-153, -166, and -190 [14]. PBDEs

congeners also showed estrogenic potencies in human T47D breast cancer cells. It has been reported that BDE-99 dose-dependently reduced the magnitude of IGF-I mRNA induction by E_2, and increased the magnitude of ER beta repression in rats[15]. Study shows that no significant ($p>0.05$) induction of CYP 1A1/EROD was observed for either individual or mixed PBDEs. The EC_{50} values for Ah receptor binding of individual PBDEs congeners were in the μM range, indicating a weaker affinity than for the reference toxicant TCDD. These results also indicate that most PBDEs congeners and commercial Penta-BDE and Deca-BDE mixtures were inactive and antagonized the action of TCDD as already reported [8].

The toxic and biochemical responses of dioxin-like compounds are primarily mediated by the aryl hydrocarbon (Ah) receptor. In general, exposure to rat H4EII cells to the positive control (TCDD) resulted in a TCDD induced CYP 1A1 activity and AhR-EGFP reported gene expression [13]. The toxicity of PCB is highly dependent on their molecular structure. For example, PCB congeners with para- and meta- but without ortho positions possess a nearly coplanar conformation and similar molecular dimension as TCDD (Peters et al., 2004). Similar to BDE-77, -119, and -126 were moderately active towards dioxin response element (DRE) binding and induced responses of both CYP 1A1 mRNA and CYP 1A1 protein equivalent to the maximal response of TCDD in primary rat hepatocytes, although at concentrations three to five orders of magnitude greater than TCDD [8]. Results of another study demonstrated that BDE–100, –153, and –183 were very weak activators for DRE binding[5].

In H4IIE, some of the individual PBDEs congeners acted via the AhR signal transduction pathway as agonists, antagonists, or both [3]. It has been indicated that the PBDEs congeners (BDE-77 and BDE-126) analogous to coplanar PCBs had a larger relative binding affinity than those with two or more ortho-bromines, such as BDE-71, -100, -153, and -154. PBDEs congeners and commercial PBDEs mixtures had Ah receptor binding affinities 10^{-2} to 10^{-5} times that of TCDD. Their potencies were approximately six orders of magnitude lower than that of TCDD but comparable to those of some mono-ortho PCBs such as PCB-105 and PCB-118 [16]. Interestingly, binding affinities for PBDEs could not be related to the planarity of the molecule, possibly because of sterical reasons [3].

The results of the EROD in vitro test indicate that BDE–209 (with their metabolisms) was absorbed into liver (Fig.1). Detailed information on Japanese medaka exposed to BDE-209 under the flow-through system can be found by Luo et al (not published). Results indicate BDE-209 absorbed into liver have a weak Ah receptor. The EROD results were significantly ($p<0.05$) influenced by the exposure time and concentration. Medaka liver cell was affected in all treatment groups for 15 days under the flow-through exposure system. Previous studies showed that BDE-47 caused three-fold induction of EROD after 14 days exposure (18 mg/kg ww)[7]. No dioxin-like toxicity was found for BDE-47, -85, and -99 in rainbow trout early life stage mortality assays [3].

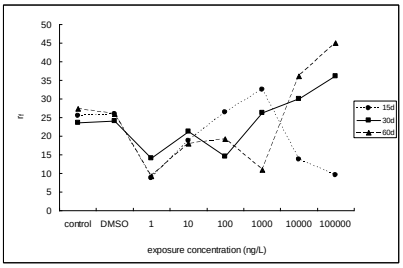

Fig.1 EROD results (in vivo) in liver of Japanese medaka exposed to BDE–209 with different concentrations under the flow-through exposure system at 15, 30, and 60 days.

SOS/umu Assays indicated that ***β***-galactosidase activities were not significantly ($p>0.05$) induced when exposed to 0.1 and 10 μM of PBDEs (7 individual congeners and commercial Penta-BDEs product DE–71) in HII4E cell ($1<IR<2$). As with the commercial PCBs mixtures, the commercial Deca-, Octa-, and Penta-BDE mixtures were not mutagenic in *S. typhimurium.* It was also noted that octa-BDEs and BDE–209 did not cause any effects in Salmonella mutagenicity tests and unscheduled DNA synthesis assay[12]. However, after exposing hapatocytes to PBDEs in combination with 4-NQO (a positive control), a concentration-dependent decrease in 4-NQO induced SOS/*umu* activity was observed. A decrease of 4-NQO was different with PBDEs exposure concentration. All PBDEs congeners showed a similar reduction though quantitative differences were observed. Fig.1 also shows that ***β***-galactosidase activity in SOS/*umu* assays when exposed to 0.1 μM of BDE-28 and BDE-183 in combination with 4-NQO was higher than those to 10 μM. ***β***-galactosidase activities of BDE-209 at

0.1 μM were lower than that at 10 μM. For BDE-47,-99, -153, and -154 and DE–71 exposed to different concentrations of 4-NOQ, their combination activities were at 0.1 μM lower or higher than those at 10 μM.

SOS/*umu* activities of BDE-28, -99, -153, and -154 exponentially increased with 4-NQO concentration ranging from 50 to 200 μg/mL then slowly increased from 200 to 350 μg/mL, reaching a steady state. Similar to individual 4-NQO exposure, SOS/umu activity of 4-NQO in combination with BDE-153, -154, and -183 exponentially increased from 50 to 150 μg/mL of 4-NQO then slowly increased from 150 to 250 μg/mL, but decreased from 250 to 350 μg/mL.

All PBDEs congeners showed a similar reduction in SOS/*umu* assays, though quantitative differences were observed. In addition, the results showed that β-galactosidase activity decreased after exposure to PBDEs in combination with 4-NQO. A concentration-dependent decrease in 4-NQO induced β-galactosidase activity was observed. β-galactosidase activity increased after exposure to PBDEs (BDE-209, BDE-183 and DE-71 at 0.1 μM) in combination with 4-NQO at a concentration 75 μg/mL higher than that exposed to 4-NQO or individual PBDEs. Generally, β-galactosidase activity at concentration 10 μM was higher than at 1 μM such as when exposed to BDE-47, -99, and -153 with 4-NQO as from 125 to 200 μg/mL 4NQO and to BDE-154 with 4-NQO as lower than 150 μg/mL 4-NQO. ***β***-galactosidase assay was developed and became a useful and valuable tool allowing detection of potential chemical mutagens and carcinogens. It was demonstrated that the umu operon in Escherichia coli that was responsible for chemical mutagenesis [17]. The present results indicated that BDE-209, BDE-183 and DE-71 at 0.1 μM have potential genotoxicity/mutagecity while other PBDEs congeners have potential antigenotoxicity/antimutageticity.

4. Conclusion

No significant estrogenic effect was observed for individual PBDEs (including BDE-28, -47, -99, -153, -154, -183, and -209) and commercial product DE–71 based on recombinant yeast bioassay. A weak binding affinity of PBDEs congeners to the Ah receptor and the weak induction of EROD activity were found. No significant mutagenicity and carcinogenicity for individual PBDEs (1<IR<2) was observed based on the SOS/*umu* assays; β-galactosidase activity decreased through exposure to PBDEs in combination with 4-NQO at concentrations from 50 to 300 μg/mL (IR>=2 as 4-NQO is 50 μg/mL). The present results indicate that PBDEs have a weak or moderate binding affinity to the receptor (estrogenic or EROD) and induce the umu operon. They are inactive at all stages of signal transduction and expression. These results indicate that PBDEs could have potential genotoxicity and antigenotoxicity. Further toxicity assays are needed in order to provide genotic/antigenotic and mutagetic/ antimutagetic reaction pathways in vitro (cell line and bacteria) and in vivo.

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