Endocrine effects of methoxylated brominated diphenyl ethers in three in vitro models

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Abstract

Methoxylated brominated diphenyl ethers (MeO-BDEs) in aquatic environments have been found to be primarily of natural origin in the marine environment and not from biotransformation of synthetic PBDEs. Two of the eight MeO-PBDEs (2-MeO-BDE-68 and 6-MeO-BDE-47) that were detected in anchovy from the Yangtze River Delta, were natural products from marine organisms. So 2-MeO-BDE-68 and 6-MeO-BDE-47 were chosen to study the potential to modulate androgen, estrogen, or thyroid hormone receptor- (AR, ER, ThR) mediated responses by use of reporter gene assays. 2-MeO-BDE-68 was antiandrogenic at 50 μM, estrogenic at 10 μM and antiestrogenic at 10 and 50 μM (IC50 = 4.88 μM). 2-MeO-BDE-68 enhanced luciferase expression by 5 nM T3 at 50 μM. 6-MeO-BDE-47 exhibited potent antiandrogenicity at 1 μM and greater (IC50 = 41.8 μM) and possessed estrogenic activity at 10 μM and antiestrogenic activity at 10 and 50 μM (IC50 = 6.02 μM).

1. Introduction

Methoxylated brominated diphenyl ethers (MeO-BDEs), which are structurally related to PBDEs, have been known for decades (Sharma and Vig, 1972; Carté and Faulkner, 1981). Polychlorinated diphenyl ethers (PBDEs) are commonly used as flame retardants in construction materials, textiles, and polymers in electronic equipment (Alaee et al., 2003; Brown et al., 2004). They have been widely found in soil, sediment, water, and air (Alaee et al., 2003; biological samples (fish, seashell, birds, earth mammals and sea mammals) (Lindström et al., 1999; Johnson and Olson, 2001; Wolkers et al., 2004), as well as in human blood serum, adipose tissue, breast milk, placental tissue and the brain (Sjödin et al., 2004; Athanasiadou et al., 2008; Domingo et al., 2008). Concentrations of PBDEs in the environment are increasing (Sellström et al., 2004; Meironytè et al., 1999; She et al., 2002; Schecter et al., 2005).

Recently MeO-BDEs have drawn more and more attention because they have been identified to be bioaccumulated by biota (Haglund et al., 1997) in top predators, such as polar bears (Ursus maritimus) from Norwegian Arctic (Verreault et al., 2005), whales from Virginia (Teuten et al., 2006), whales and dolphins from the Mediterranean Sea (Pettersson et al., 2004; Teuten et al., 2006), sea lions (Zalophus californianus) from California (Stapleton et al., 2006), cetaceans from Australian waters (Vetter et al., 2002; Melcher et al., 2005), pike from Swedish waters (Kierkegaard et al., 2004), fish and guillemot from Baltic, Atlantic and Arctic environments (Sikkonen et al., 2004), pinnipeds from the Baltic Sea (Haglund et al., 1997), harbor porpoises and harbor seals from the North Sea (Wejsj et al., 2009a–c), marine mammals from Japan (Marsh et al., 2005) and beluga whales from the Canadian Arctic (Kelly et al., 2008). The distribution of MeO-BDEs is ubiquitous at concentrations sometimes greater than those of PBDE congeners (Teuten et al., 2005). Since MeO-BDEs are neither a commercial product nor reported to be byproducts in industrial processes (Vetter, 2006), it had been postulated that Meo-BDEs could be formed by direct methoxylation or in a two-step process by a hydroxylation followed by a methylation, either of which is a viable biochemical transformation (Haglund et al., 1997; Hakk and Letcher, 2003). It had been suggested that MeO-BDEs might be biotransformation products of PBDEs, possibly through hydroxy-BDEs (HO-BDEs) intermediates. The pathways of formation have been elucidated and it is now evident that some MeO-BDEs are not formed from HO-BDEs and PBDEs but rather that the MeO-BDEs are natural products, especially in the marine environment (Teuten et al., 2005; Wan et al., 2009; Su et al., 2010). MeO-BDEs are

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bioransformed to HO-BDEs and in fact are the primary source of HO-BDEs and little of the HO-BDEs formed from synthetic PBDEs (Wan et al., 2010a,b).

Nuclear hormone receptors are ligand-dependent transcription factors that regulate a variety of important physiologic processes (McKenna et al., 1999). Several studies have reported that PBDEs can disrupt the endocrine system via a variety of nuclear hormone receptors, such as estrogen receptor (ER) and androgen receptor (AR) (Meerts et al., 2001; Stoker et al., 2005; Hamers et al., 2006). In the ER-CALUX assay using a human T47D breast cancer cells, the ER was activated by less-brominated PBDEs and inactivated by more-brominated PBDEs (Meerts et al., 2001; Hamers et al., 2006). Consistent with these findings is the recent observation that BDE-28, -47, and -100 were estrogenic while BDE-99 and -100 were antiestrogenic to Chinese hamster ovary (CHO-K1) cells (Kojima et al., 2009). Although none of the tested PBDE congener showed any androgenic activity, BDE-100 has been reported to be an antiandrogen (Stoker et al., 2005). Some PBDEs are AR antagonists (Hamers et al., 2006; Kojima et al., 2009). PBDEs bind competitively with human transthyretin (TTR), a transport protein for the thyroid hormones triiodothyronine (T3) and thyroxine (T4), thereby affecting transport of thyroid hormone (Meerts et al., 2000; Zhou et al., 2001; Richardson et al., 2008). No PBDEs have been reported to bind to the thyroid hormone receptor (Thr) (Kitamura et al., 2008).

There is little information on the toxicology of MeO-BDEs, especially their endocrine disrupting properties. Since MeO-BDEs are structurally similar to PBDEs which have the potency to disrupt the balance of endocrine system, and are present in relatively great concentrations in marine organisms, including food eaten in Asia (Wan et al., 2010b) it was decided to determine if MeO-BDEs can affect endocrine homeostasis. In order to test this hypothesis, endocrine modulating potential of 6-MeO-BDE-47 and 2′-MeO-BDE-68, which are most frequently observed MeO-BDEs in animal tissue (Kierkegaard et al., 2004; Teuten et al., 2006), were assessed.

Concentrations of MeO-PBDE in dolphins of the continental shelf (CS) of Brazil are among the greatest detected in cetaceans (up to 250 μg/g lw) (Dorneles Paulo et al., 2010). The greatest concentration of 2′-MeO-BDE-68 previously reported for marine mammal tissues was 3760 ng/g wet weight found in the blubber of a pygmy sperm whale from Queensland, northeast Australia (Vetter et al., 2002). The lipid content of blubber in marine mammals ranges from 30% to 90% (Reinders and Aguilar, 2002). Considering the lesser of the lipid percentages (30%) a maximum value of 12,500 ng/g lw can be calculated for 2′-MeO-BDE-68 in the pygmy sperm whale. Concentrations of the sum of MeO-triBDEs were less, with a maximum value (260 ng/g lw) determined in a false killer whale (Pseudorca crassidens), which contained also the greatest concentrations of 2′-MeO-BDE-68 and 6-MeO-BDE-47 (sum 250 μg/g lw) (Dorneles Paulo et al., 2010). 6-MeO-BDE-47 and 2′-MeO-BDE-68 were two out of eight MeO-PBDEs, which were detected in Ancho- vy (Colia sp.) from the lower Yangtze River in the region around Jiangsu Province of China (Su et al., 2010).

Promoter-reporter gene assays have been used as in vitro methods for clarifying agonistic and antagonistic potency of various chemicals against nuclear hormone receptors. AR-, ER-, and ThR-mediated transactivation reporter gene assays were used to assess antiandrogenic, anti/estrogenic and anti/thyroid hormone properties of these two MeO-BDEs.

2. Materials and methods

2.1. Chemicals

5α-Dihydrotestosterone (DHT), 17β-estradiol (E2) with purities of over 99% were purchased from Sigma Chemical Co. (St. Louis, MO, USA). L-3,5,3′-triiodothyronine (T3) with a purity of over 98% was also purchased from Sigma Chemical Co. (St. Louis, MO, USA), 2′-MeO-BDE-68 and 6-MeO-BDE-47 (Fig. 1) were synthesized in the Department of Biology and Chemistry of City University of Hong Kong and have been confirmed to have purities of greater than 98% (Wan et al., 2010a). Stock solutions of the chemicals were prepared in dimethyl sulfoxide (DMSO, Sigma), stored at −20 °C, and diluted to desired concentrations in phenol red-free Dulbecco’s modified Eagle medium (DMEM, Sigma) or red-free L-15 medium (Sigma) immediately before use.

2.2. Plasmids

The luciferase reporter plasmid pERE-TATA-Luc and rat estrogen receptor α (ERα) expression vector ERαs/pCI were provided by Dr. Takeyoshi (Chemicals Assessment Center, Chemicals Evaluation and Research Institute, Oita, Japan). The plasmids were constructed as previously described (Takeyoshi et al., 2002). The expression plasmid pGal4-L-TRδ and Gal4 responsive luciferase reporter plasmid pUSAS-tk-Luc containing four copies of the Gal4 binding site were obtained from Professor Ronald M. Evans (Gene Expression Laboratory, Howard Hughes Medical Institute, San Diego, CA, USA), and their structures had been described before (Chen and Evans, 1995).

2.3. Luciferase reporter gene assay

2.3.1. AR reporter gene assay

MDA-kb2 cells (ATCC, USA), stably transformed with murine mammalian tumor virus (MMTV)-luciferase were cultured in Leibovitz’s L-15 medium with 10% fetal bovine serum (FBS, Gibco), 100 U/ml penicillin (Sigma), 10 μg/ml streptomycin (Sigma), and 0.25 μg/ml amphotericin B (Sigma) at 37 °C without CO2. The MMTV is not specific for androgen. However, this stable cell line has been used for androgen screening (Wilson et al., 2002). Cells were plated at 1 × 104 cells per well in 100 μl of medium in 96-well luminometer plates. When cells were attached (4–6 h), medium was removed and replaced with dosing medium, which contained the test chemicals, including MeO-BDEs and or model chemicals of known potency. The MDA-kb2 cells were exposed to DHT (Sigma, 1.0 × 10−11–1.0 × 10−6 M in 10-fold dilution steps), solvent-controls or test chemicals for 24 h. The model compounds were tested at a concentration range that had been shown to be noncytotoxic. After rinsing three times with phosphate-buffered saline (PBS, pH 7.4), cells were lysed with 1 × passive lysis buffer (Promega). After centrifuging at 12,000g for 10 min to remove debris, luciferase activities in cell lysates were analyzed immediately using a 96-well plate luminometer (Berthold Detection System, Pforzheim, Germany). The amount of luciferase was measured by use of the luciferase reporter assay system kit (Promega) following the manufacturer’s instructions.
elements (HRE), E2 and T3 cannot induce Luc activity. CV-1 cells were from monkey kidney, which do not contain the endogenous receptors (ER and Thr). CV-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 5% FBS, 100 U penicillin/ml and 100 streptomycin μg/ml, at 37 °C in an atmosphere containing 5% CO₂. Cells were cultured and exposed according to previously published methods (Sun et al., 2008a,b). Briefly, host cells were plated in 48-well microplate at a density of 0.5 × 10⁵ cells per well in DMEM medium containing 5% charcoal-dextran-stripped FBS that was free of phenol red (CDS-FBS). After 12 h cells were transfected. The concentration of plasmids and transfection reagent for ER activity and ThR activity was described before (Zhang et al., 2011). After an incubation period of 12 h, cells were treated with test chemicals for 24 h to E2 (1.0 × 10⁻¹⁰–1.0 × 10⁻⁷ M in 10-fold dilution steps), solvent-controls and compounds. Then, luciferase activity was determined as described above.

2.3.3. ThR reporter gene assay
CV-1 cells were cultured and plated as described above. After an initial incubation of 12 h, cells were transfected. After an incubation period of 12 h, cells were exposed to T3 (1.0 × 10⁻¹²–1.0 × 10⁻⁸ M in 10-fold dilution steps), solvent-controls and test chemicals for 24 h. Cell lysates were analyzed immediately using a 96-well plate luminometer (Berthold Detection System, Pforzheim, Germany).

2.4. Cell viability assessed by MTT assay
The MTT assay was performed to detect the cytotoxicity of test chemicals. Both MDA-kb2 and CV-1 cells attached to culture dishes were collected and plated at a density of 1 × 10⁴ cells/100 μl in each well of 96-well plates using DMEM with 5% DCC serum and L-15 with 10% DCC serum, respectively. After 24 h, cells were treated with vehicle or various concentrations (0.1, 1, 10 and 50 μM) of test chemicals alone or with 1.0 nM DHT, 1.0 nM E2, or 5.0 nM T3 for 24 h. Then, 25 μl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml in PBS, Sigma) was added to each well and continuously incubated at 37 °C for 4 h, after which MTT solutions were replaced with 150 μl DMSO and shaken for 10 min to solubilize crystals. Absorbance was measured by automatic microplate reader (EL808, Bio-Tek, Winooski, VT, USA) at 570 nm.

2.5. Statistical analysis
All experiments were conducted in triplicate and within an experiment; each concentration was tested in triplicate. Values are reported as the mean ± standard deviations (SD) (n = 3). Results were analyzed by one-way analysis of variance (ANOVA), followed by Duncan’s multiple comparisons test (SPSS 11.5; SPSS Inc., Chicago, IL, USA). The level of significance was set at p < 0.05. For agonists, treatments were compared to the vehicle control group, and the relative transcriptional activity was converted to fold induction relative to that of the vehicle control. For antagonists, treatments were compared to the activity caused by 1.0 nM DHT, 1.0 nM E2 or 5.0 nM T3 as positive controls, and percent of the activity caused by 1.0 nM DHT, 1.0 nM E2 or 5.0 nM T3. The median inhibitory concentration (IC₅₀) or median effective concentration (EC₅₀) was calculated with SPSS software (SPSS Inc., Chicago, IL, USA).

3. Results
3.1. Cell viability and responsiveness
There was no statistically significant difference between vehicle-treated groups and those treated alone or with 1.0 nM DHT, 1.0 nM E2, 5.0 nM T3 (data not shown) for neither MDA-kb2 nor CV-1 cells. No cytotoxicity was observed at the concentrations of chemicals tested. The assays displayed appropriate responses to the known androgen receptor (AR) agonist 5α-dihydrotestosterone (DHT), estrogen receptor (ER) agonist estradiol (E2) and thyroid hormone receptor (ThR) agonist triiodothyronine (T3). The method responsiveness was described before (Zhang et al., 2011).

3.2. Antiandrogenicity
Chemicals were tested for their inhibitory effects on transcriptional activity induced by co-exposure to DHT. 2'-MeO-BDE-68 did not reduce luciferase expression at concentrations from 0.1 to 10 μM, but significantly less luciferase activity was observed at 50 μM. The IC₅₀ for 2'-MeO-BDE-68 was 41.8 μM (Fig. 2). 6-MeO-BDE-47 was a potent antiandrogen that significantly inhibited the luciferase activity induced by 1 nM of DHT at concentration of 1 μM and greater (Fig. 2). The IC₅₀ value of 6-MeO-BDE-47 was 4.3 μM. 6-MeO-BDE-47 was a stronger antagonist than 2'-MeO-BDE-68.

3.3. Estrogenic and antiestrogenicity
Neither 2'-MeO-BDE-68 nor 6-MeO-BDE-47 resulted in induction of luciferase activity that was greater than that of the vehicle control at 0.1 or 1.0 μM, while both of them displayed weak estrogenic potency with maximal induction of 1.47- and 1.58-fold, respectively at a concentration of 10 μM (Fig. 3).
Neither 0.1 nor 1.0 μM of either 2'-MeO-BDE-68 or 6-MeO-BDE-47 resulted in less expression of luciferase in the presence of E2 (Fig. 4). Concentrations of both 2'-MeO-BDE-68 and 6-MeO-BDE-47 greater than 10 μM significantly antagonized the effect of E2 on ER-mediated luciferase activity with IC₅₀ values of 4.88 and 6.02 μM, respectively.

3.4. Thyroid and antithyroid potencies
Effects of the two MeO-BDEs on ThR-mediated luciferase activity were determined in the presence of 5.0 nM T3. While
Antiestrogenic potency of 2'-MeO-BDE-68 and 6-MeO-BDE-47 (Fig. 5). Neither of the two MeO-BDEs exhibited ThR agonistic activity at concentrations from 0.1 to 10 μM (Data not shown).

50 μM, 2'-MeO-BDE-68 significantly increased luciferase activity (Fig. 5), no significant antagonistic activity was detectable at the tested dosages for 6-MeO-BDE-47 (Fig. 5). Neither of the two MeO-BDEs exhibited ThR agonistic activity at concentrations from 0.1 to 10 μM (Data not shown).

Fig. 3. Estrogenic potency of E2, 2'-MeO-BDE-68 and 6-MeO-BDE-47 measured by use of the CV-1 reporter gene assay. Estrogenic potency is reported as expression reporter gene activity relative to that of untreated cells (control) (mean ± SD) of three independent experiments. Significant differences are indicated by asterisks *, P < 0.05 vs. control.

Fig. 4. Antiestrogenic potency of 2'-MeO-BDE-68 and 6-MeO-BDE-47 measured by reporter gene assay with CV-1 cells. These chemicals were added along with 1 nM E2. Data were (mean ± SD) of three independent experiments and are presented as percent induction, with 100% activity defined as the activity achieved with T3 (5 nM). Significant differences are indicated by asterisks *, P < 0.05 vs. the value of 5 nM T3 (100%).

4. Discussion

Transactivation, reporter gene assays are useful to characterize receptor-mediated endocrine activity and elucidate mechanisms of action (Freyberger and Schmuck, 2005). In the present study, three reporter gene assays were used to investigate potency of two PBDE methoxylated derivatives to modulate AR-, ER- and ThR-mediated responses. Both MeO-BDEs possessed mixed antiandrogenic activity, estrogenic/antiestrogenic activities.

A novel stable cell line, MDA-kb2, was used to determine the potency of androgen antagonists. The assay was specific and sensitive to AR antagonistic chemicals. Maximal induction of about 9-fold relative to that of vehicle control has been reported to occur at 10 nM DHT (Wilson et al., 2002). The assay displayed appropriate sensitivity to the known AR agonist, DHT. The maximal inductions of 11.2-fold relative to vehicle control which was slightly greater than which had been previously reported (Wilson et al., 2002), but exhibited a similar dose–response relationship. Detection of androgen antagonist activity of compounds was done by measuring their ability to decrease DHT-induced luciferase activity. In studies upon which we report here, concentrations of DHT within the linear concentration–response range were used. Antagonist activity can usually be detected in competition against 1.0 nM DHT, which is equivalent to the concentration observed in men of 20–40 years of age (Lewis et al., 1976), and can induce an 8.08-fold change in luciferase activity. In vitro antiandrogenicities of MeO-BDEs have been reported previously (Kojima et al., 2009). The results of that study indicated that, all MeO-BDEs tested were potent antiandrogens.

Both estrogenic and antiestrogenic potencies of MeO-BDEs were determined by use of a sensitive reporter gene assay based on CV-1 cells, which had been transiently transfected with expression vectors for ER-α (rERα) along with a plasmid encoding for the reported gene, luciferase. While the sensitivity of this system has been demonstrated previously (Sun et al., 2008b), in this study a maximum fold-change of 17.02-fold relative to the vehicle control was observed. In the present study, 2'-MeO-BDE-68 and 6-MeO-BDE-47 were weakly estrogenic with 1.47-, and 1.58-fold greater ER-mediated luciferase activity relative to the vehicle control. Both MeO-BDEs antagonized the ERα at greater doses (10 and 50 μM) in the presence of 1.0 nM E2. Taken together, the results suggested that MeO-BDEs might act as ERα agonists and/or antagonists, depending on concentration, which is similar to the results observed previously (Kojima et al., 2009). However, all previous studies were
conducted with para-MeO-BDEs (Kojima et al., 2009). Until now there was no information about ortho-MeO-BDEs. Our results also indicated that some MeO-PBDEs exhibit agonistic and antagonistic interactions with the ER.

PBDEs and HO-BDEs can bind competitively to human transthyretin (TTR), a transport protein for the thyroid hormones T3 and thyroxine (T4), thereby interfering with functioning of thyroid hormone (Meerts et al., 2001; Zhou et al., 2001, 2002; Richardson et al., 2008). However, few studies have addressed the effects of MeO-BDEs on thyroid hormone function except for the results for reporter gene assays using Chinese hamster ovary cells by (Kojima et al., 2009).

6-MeO-BDE-47 exhibited endocrine disrupting effects similar to those of BDE-100, which is structurally similar, with the only difference being that the methoxy group on 6-MeO-BDE-47 is replaced by a bromine atom in BDE-100. In vivo and in vitro antiandrogenicity of BDE-100 have been reported previously (Stoker et al., 2005; Hamers et al., 2006; Kojima et al., 2009). Alternatively BDE-100 can antagonize the effect of E2 (Hamers et al., 2006; Kojima et al., 2009), but was not an agonist or antagonist of T3 (Kojima et al., 2009). This result indicates that MeO-BDEs have endocrine-disrupting potential similar to those of PBDEs.

MeO-BDEs act as agonist and/or antagonist via ERα and AR in vitro reporter gene assays. Based on these results, the predominant MeO-BDEs found in human tissues have multiple endocrine-disrupting effects modulated via nuclear hormone receptors. Ligand-dependent transcription of nuclear hormone receptors requires association of protein cofactors and basal transcription factors, expression levels of which differ from cell to cell (McKenna et al., 1999). Furthermore, there is some discrepancy in the cellular metabolic ability against different cells. Thus, there is a possibility that different cells could respond differently. The CV-1 and MDA-kb2 cells used in our study were deficient in metabolic activity, especially compared with hepato carcinoma cells which have some metabolic activity that could affect the test result. Thus, the results of our study reflected the effects of 2’-MeO-BDE-68 and 6-MeO-BDE-47 and not their biotransformation products.

Most of the effects observed in this study occurred at concentrations of 10 or 50 μM, which is greater than relevant concentrations that have been observed in humans and the environment. In addition, the results of previous studies have suggested that MeO-BDEs have the potential to interfere with CYP17 and CYP19 activities, both of which catalyze key steps in the production of sex hormones in humans (Cantón et al., 2005, 2006; He et al., 2008; Song et al., 2008). Therefore, MeO-BDEs may also indirectly disrupt the endocrine system by affecting the expression of relevant genes other than interacting with nuclear hormone receptors. Furthermore, information about the potential endocrine activity of MeO-PBDEs is limited to several MeO-BDEs (Song et al., 2008; Kojima et al., 2009).

5. Conclusions

Few studies have been done to characterize the endocrine-disrupting potential of MeO-BDEs, some of which are natural products especially in marine organisms that are consumed by humans. In the present study, the antiandrogenic activity of two frequently detected MeO-BDEs (2’-MeO-BDE-68 and 6-MeO-BDE-47) was determined by use of a stably transfected cell line (MDA-kb2). The (anti)estrogenic and (anti)thyroid activity was determined by use of the rat estrogen receptor α (rERα) and human thyroid hormone receptor (hThR) mediated luciferase reporter gene assay in African green monkey kidney cells (CV-1). The assays displayed appropriate responses to the known androgen receptor (AR) agonist 5α-dihydrotestosterone (DHT), estrogen receptor (ER) agonist estradiol (E2) and thyroid hormone receptor (Thr) agonist triiodothyronine (T3). 2’-MeO-BDE-68 was antiandrogenic only at 50 μM, but was estrogenic at 10 μM and antiestrogenic at 10 and 50 μM. 2’-MeO-BDE-68 was found to enhance luciferase expression by 5 nM T3 at 50 μM. The result also showed that 6-MeO-BDE-47 exhibited potent antiandrogenic activity at the concentration of 10 μM and higher. 6-MeO-BDE-47 possessed estrogenic activity at 10 μM and antiestrogenic activity at 10 and 50 μM. These results suggest that these two MeO-BDEs can cause multiple endocrine-disrupting effects through interfering with several hormonal signaling pathways simultaneously.

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