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Structure-dependent activities of polybrominated diphenyl ethers and hydroxylated metabolites on zebrafish retinoic acid receptor

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Abstract Polybrominated diphenyl ethers (PBDEs), a group of potential endocrine-disrupting chemicals (EDCs) have been shown to disrupt retinoid homeostasis in different species in both laboratory and field studies. However, the molecular mechanisms of interactions with the retinoic acid receptor (RAR) are not fully understood. Zebrafish have proven useful for investigating mechanisms of chemical toxicity. In the present study, a reporter gene assay was used to investigate the activities of 11 PBDEs and six OH-PBDEs with different degrees of bromination on zebrafish RAR. All tested OH-PBDEs induced RAR transcriptional activity; however, of the 11 PBDEs examined, only BDE28 and BDE154 affected the RAR transcriptional activity. Homology modeling and molecular docking were employed to simulate the interactions of PBDEs/OH-PBDEs with zebrafish RARs and to identify binding affinities to analyze the specialization of the interaction between RARs and PBDEs/OH-PBDEs. The results showed that although these compounds could bind with RARs, the effects of PBDEs/OH-PBDEs on RAR transcriptional activity did not depend on their RAR-binding abilities. The present study is the first attempt to demonstrate that OH-PBDEs could induce RAR transcriptional activity by binding directly with RAR; these effects are possibly related to the structure of the compounds, especially their hydroxylation and bromination. Most of the PBDEs could not directly interact with the RAR.

Keywords PBDEs · OH-PBDEs · Retinoic acid receptor · Reporter gene assay · Homology modeling · Molecular docking

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Introduction

Brominated flame retardants (BFRs), such as polybrominated diphenyl ethers (PBDEs), have been commonly used in households globally over the past decades. PBDEs are potential endocrine-disrupting chemicals (EDCs) (Darnerud et al. 2001). PBDEs and their hydroxylated (OH−) or methoxylated (MeO−) metabolites have been detected in human and wildlife (Lacorte and Ikonomou 2009; Zhang et al. 2010). OH-PBDEs have been reported to originate from both the biotransformation of PBDEs/MeO-PBDEs and as the natural products of some marine invertebrates (Wan et al. 2009; Wiseman et al. 2011). The presence of OH-PBDEs is of concern because of their higher toxic potencies relative to PBDEs (Meerts et al. 2001; Hamers et al. 2006, 2008; Dingemans et al. 2008). Moreover, OH-PBDEs have been detected in humans in concentrations similar to or even higher than those of parent PBDEs (Athanasiadou et al. 2008; Qiu et al. 2009). There is increasing evidence that exposure to PBDEs might result in endocrine disruption and neurotoxicity (Dingemans et al. 2011; Stapleton et al. 2011). To date, most studies have focused on the disruption of the thyroid hormone (TH) system since the chemical structures of PBDEs and their metabolites, especially OH-PBDEs, are similar to that of THs (Richardson et al. 2008; Schreiber et al. 2010). In addition, numerous studies have shown that retinoid homeostasis was sensitive to the exposure of PBDE congeners and commercial mixtures

in different species including rodents, birds, and fish (Cesh et al. 2010; Ellis-Hutchings et al. 2006, 2009; Hallgren et al. 2001; Fernie et al. 2005; van der Van et al. 2008; Chen et al. 2012; Xu et al. 2013).

Despite the susceptibility of retinoid homeostasis to PBDE exposure in different species that has been reported in both laboratory and wildlife studies, the molecular mechanisms of interactions with the retinoic acid receptor (RAR) remain largely unknown. Retinoic acid (RA) is the oxidized and bioactive form of retinol (vitamin A). RA signaling has a critical function in various developmental processes in vertebrates (Duester 2008; Kam et al. 2012; Niederreither and Dolle 2008; Zile 2001). The RA signaling pathway is mediated by the RAR and retinoid X receptor (RXR). After transport into the nucleus by cellular RA binding proteins (CRABPs), RA binds to heterodimers of RAR and RXR (RAR:RXR), and this activated complex binds to RA responses elements (RAREs) in the promoter region of target genes, causing the release of corepressors and the recruitment of coactivators, which leads to the activation of transcription of target genes (Niederreither and Dolle 2008; Theodosiou et al. 2010). A previous study showed that PBDE exposure altered the expression levels of genes involved in RA signaling, such as cellular retinoic acid-binding proteins (*crabp1a*, *crabp2a*) and the retinoic acid receptor subunit (*raraa*) in zebrafish larvae (Xu et al. 2013), indicating that PBDEs might alter RA signaling.

Several toxicological studies have suggested that the toxicity of PBDEs results from a receptor-mediated effect. Toxic effects of PBDEs appear to be related to the number of bromines present. In a previous investigation of the effects of PBDEs on embryonic development of zebrafish, researchers found that there was a positive relationship between logK_{ow} and adverse effects of six PBDEs (including alteration in behavior, physical malformation, and mortality): the lower brominated congeners were more toxic, while higher brominated congeners did not elicit an observable effect (Usenko et al. 2011). In estrogen receptors (ERs) transcription cell assay, low-brominated OH-PBDEs acted as ER agonists, whereas high-brominated compounds exhibited antagonistic activity (Li et al. 2013). Second, the toxic effects are related to the position of substituents, such as bromine and hydroxyl groups. For example, both BDE99 and BDE100 are penta-congeners. Through switching the bromine from the para-position of BDE99 to the ortho-position, thereby forming BDE100, BDE99 resulted in a greater rate of zebrafish embryo mortality than BED100 at 13 mg/L (Usenko et al. 2011). Moreover, the effect of OH-polychlorinated biphenyls (OH-PCBs) on steroid hormone receptors such as the ERα/β, androgen receptor (AR), and glucocorticoid receptor (GR), depended on the position of the OH-groups (Takeuchi et al. 2011). Third, the toxic effects are dependent on the degree of hydroxylation. Recent studies have demonstrated that the toxic effects of PBDEs might be, in part, due to their OH metabolites (Dingemans et al. 2008; Wiseman et al. 2011). Additionally, since only a limited number of PBDEs/OH-PBDEs have been investigated, there is uncertainty regarding the role of compound structure on structural interpretation concerning the differences in toxicity and the related molecular mechanisms of PBDE congeners.

In this study, a reporter gene assay was conducted to investigate the activities of 11 PBDEs and six OH-PBDEs with different degrees of bromination on zebrafish RAR. We further employed homology modeling and molecular docking to simulate the interactions of these compounds with the receptor to understand the structural basis of the experimentally observed activities of PBDEs/OH-PBDE.

Materials and methods

Chemicals

All 11 PBDE congeners (2,4-dibromodiphenyl ether (BDE7); 2,2′,4-tribromodiphenyl ether (BDE17); 2,4,4′-tribromodiphenyl ether (BDE28); 2,2′,4,4′-tetrabromodiphenyl ether (BDE47); 2,2′,4,5′-tetrabromodiphenyl ether (BDE49); 2,3′,4,5′-tetrabromodiphenyl ether (BDE68); 2,2′,4,4′,5-pentabromodiphenyl ether (BDE99); 2,2′,4,4′,6-pentabromodiphenyl ether (BDE100); 2′,3,4,4′,5-pentabromodiphenyl ether (BDE123); 2,2′,4,4′,5,5′-hexabromodiphenyl ether (BDE153); and 2,2′,4,4′,5,6′-hexabromodiphenyl ether (BDE154); Fig. 1) were purchased from AccuStandard (purity >99 %; New Haven, CT, USA). All six OH-PBDEs (2'-hydroxy-2,4-dibromodiphenyl ether (2′-OH-BDE7); 4'-hydroxy-2,2′,4-tribromodiphenyl ether (4′-OH-BDE17); 2′-hydroxy-2,4,4′-tribromodiphenyl ether (2′-OH-BDE28); 2'-hydroxy-2,3′,4,5′-tetrabromodiphenyl ether (2′-OH-BDE68); 4-hydroxy-2,2′,3,4′,5-pentabromodiphenyl ether (4-OH-BDE90); and 2-hydroxy-2′,3,4,4′,5-pentabromodiphenyl ether (2-OH-BDE123); Fig. 1), were a generous gift from Prof. Chen JW (purity >98 %; Dalian University of Technology, Dalian, China). All-*trans* retinoic acid was purchased from sigma Aldrich (purity ≥98 %; St. Louis, MO, USA). The standard compounds were dissolved in dimethylsulfoxide (DMSO; purity >99.9 %; Amresco, Solon, OH, USA) to prepare stock solutions, and dilutions were made from stock solutions immediately prior to use.

Luciferase reporter gene assay

To evaluate whether PBDEs/OH-PBDEs could disturb RA homeostasis at the receptor level, an in vitro luciferase assay was developed (Lassiter et al. 2002; Zhao et al. 2005). For this study, FuGENE HD (Promega, Madison, WI, USA) was used

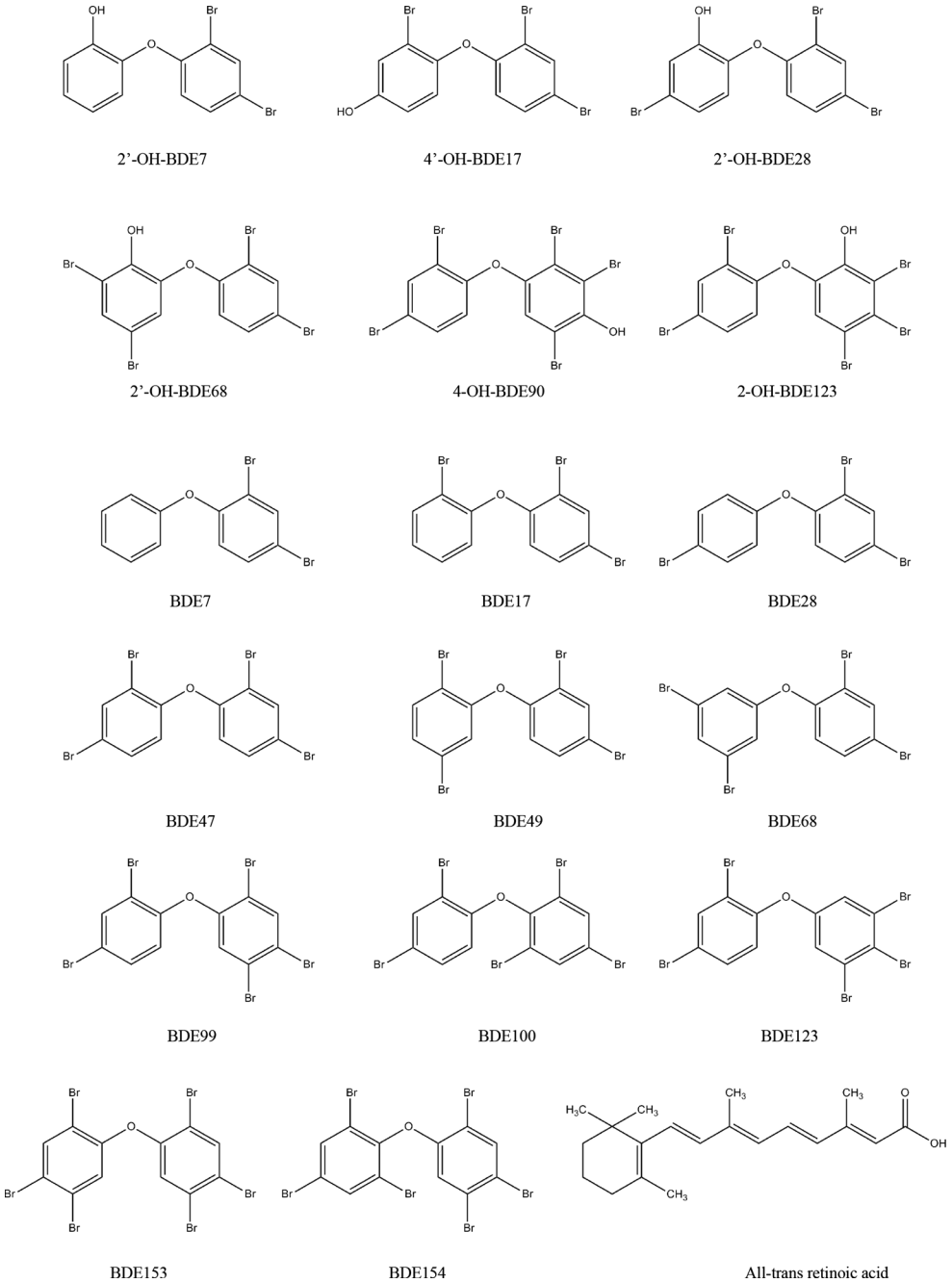

Fig. 1 Chemical structures of 11 PBDEs, 6 OH-PBDEs and all-*trans* retinoic acid (RA) investigated in the present study

as the transfection reagent and the Dual-Luciferase Reporter Assay System (Promega) was used to measure luciferase activity according to the instructions from the manufacturer.

Briefly, 293T cells (ATCC, Manassas, VA, USA) grown in 100 μL of Dulbecco's MEM (DMEM; HyClone, Logan, UT, USA) medium in 96-well plates (Corning, Cambridge, MA, USA) were transfected when they reached 70–80 % confluency. Plasmids used in this study were kindly provided by Prof. Zhao QS, Model Animal Research Center of Nanjing University, Nanjing, China. A mixture of FuGENE HD: DNA in a ratio of 3:1 at 0.1 μg DNA, including SV 40-renilla luciferase expression plasmid (Promega), pGL3-RARE reporter plasmid (Hu et al. 2008), and recombinant zebrafish RAR α expression plasmid (RAR α2.B; Perz-Edwards et al. 2001), were co-transfected into each well of 96-well plates. Transfection was done in DMEM for 24 h. Following transfection, each well was changed with 100 μL of DMEM containing 10 % stripped serum (final concentration; Biological Industries, Beit-Haemek, Israel) and the various concentrations of selected PBDEs/OH-PBDEs (5, 50, 500 μg/L) or vehicle (DMSO; the final vehicle concentration was always 0.1 % for all wells). Two hundred nanomole per

liter all-*trans* RA was used as the positive control. After 24 h of treatment, the wells were washed with cold phosphate buffered saline (PBS; Solarbio, Beijing, China) twice and the cells were lysed for dual luciferase activity assay using Passive Lysis Buffer (Promega). Luciferase activity was measured using a Luminescence Reader Synergy 2 (Biotek, Winooski, VT, USA). Each treatment was repeated three times with two independent transfections. Relative luciferase activity was expressed as the ratio of firefly luciferase activity and renilla luciferase activity, which was calculated as the indicator to determine whether the selected PBDEs/OH-PBDEs interacted with zebrafish RAR in transfected 293T cells.

Homology modeling and molecular docking

Homology modeling and molecular docking have been shown to have the potential to predict the interactions of environmental pollutants with nuclear hormone receptors (NRs) (Wu et al. 2009). In this study, homology modeling was used to construct the 3D model of ligand-binding domains (LBDs) of zebrafish RAR α and RAR γ. Molecular docking was applied to simulate binding of the selected PBDEs/OH-PBDEs to these receptors. Free energy of binding was considered as the criterion to identify the binding affinities to analyze the specialization of interactions between receptors and ligands.

Briefly, we extracted the crystal structure of human RAR α-LBD and RAR γ-LBD from the RCSB Protein Data Bank [RCSB (Research collaborator for structural bioinformatics) PDB: http://www.rcsb.org/pdb/home/home.do]. Using the online Swiss modeler server (http://swissmodel.expasy.org/), the 3D structures of zebrafish RAR α and RAR γ were constructed according to their respective human homologues. Basic information about the target and template proteins is given in Table 1.

Docking of the selected PBDEs/OH-PBDEs to the binding site of the zebrafish RARs was performed using the classical docking software AutoDock 4.2 (The Scripps Research Institute, La Jolla, CA, USA; Morris et al. 2009), which uses a Lamarckian genetic algorithm for the conformational search. Prior to molecular docking, the protein files were prepared by the removal of water molecules and other ligands, and the addition of polar hydrogens and Kollman charges. The 3D-coordinates of ligands in the PDB format were obtained through the ChemAxon (http://www.chemaxon.com) using Marvin sketch drawing. When docking, the ligands were kept rigid, while all the torsional bonds of each ligand were set free. A precalculated 3-dimensional energy grid of equally spaced discrete points was generated prior to docking for a rapid energy evaluation, using the program AutoGrid, which was included in AutoDock 4.2 (Morris et al. 1998). Docking was performed using the Lamarckian genetic algorithm. For each complex, 100 independent docking runs were conducted, and the binding mode with the lowest binding energy was chosen.

Statistical analysis

All data were expressed as mean±standard error of the mean (SEM). For the luciferase assay, statistical analyses were performed with GraphPad Prism 5 (GraphPad, San Diego, CA, USA). Since the relative luciferase activity data conformed to the assumptions of normality (Lilliefors, $p>0.05$) and homogeneity ($p>0.05$), we used one-way analysis of variance (ANOVA) followed by a post-hoc Dunnett's multiple comparison test to conduct pairwise comparisons between the control and each exposure group. For all statistical analyses, $p<0.05$ was considered statistically significant.

Results

Activity of PBDEs/OH-PBDEs on RAR determined by luciferase reporter gene assay

PBDEs and OH-PBDEs exhibited different activities on zebrafish RAR α. All tested OH-PBDEs induced RAR transcriptional activity; however, of the 11 PBDEs examined, only BDE28 and BDE154 affected the RAR transcriptional activity. Luciferase activities of both the 50 and 500 μg/L BDE154 treatment groups were significantly higher than in the control groups, while luciferase activity of the 500 μg/L BDE28 treatment was significantly lower than in the control groups (Fig. 2a, b). Luciferase activities of the other eight PBDEs were not significantly different from those of the control group. Luciferase activities of all six selected OH-PBDEs were significantly higher than those of the control group (Fig. 2c). Furthermore, relative luciferase activities of the four low-brominated OH-PBDEs (2′-OH-BDE7, 4′-OH-BDE17, 2′-OH-BDE28, and 2′-OH-BDE68) increased in a concentration-dependent manner. However, the relative luciferase activities of the two high-brominated OH-PBDEs (4-OH-BDE90 and 2-OH-BDE123) reached maximum values at a concentration of 5 μg/L, then sharply decreased at higher concentrations. Compared with the positive control, the relative luciferase activity of RA was about threefold higher than that of the selected OH-PBDEs at the same level (200 nmol/L is equal to 60 μg/L) and the maximum relative luciferase activity induced by these OH-PBDEs was lower, while the concentration at which maximum relative luciferase activity was reached was much higher (Fig. 2c).

Table 1 The basic information on the target and template proteins

Target	NCBI accession	Residues of LBD	Template		
			PDB ID	Protein	Sequence identity
zebrafish RARα	NP_571481.2	169–402	3KMR	Human RARα-LBD	91 %
zebrafish RARγ	NP_571414.1	170–407	2LBD	Human RARγ-LBD	93 %

Construction of receptor model

The sequence identity between the zebrafish RARs and their corresponding templates was greater than 90 % (Table 1). The final modeled structures of the RAR-LBDs are shown in Fig. 3.

Molecular docking of PBDEs/OH-PBDEs with RARs

The binding energy was determined from the molecular docking analysis to determine structural differences that might explain differences in the ability of OH-BDEs to interact with zebrafish RAR. The natural ligand RA was docked into zebrafish RAR α and RAR γ with favorable binding energies of −11.28 and −11.05 Kcal/M, respectively (Table 2). However, for PBDEs, the binding energies of PBDEs with RAR α (from −5.51 to −9.49 Kcal/M) and RAR γ (from −4.49 to −9.07 Kcal/M) were considerably higher than for RA (Table 2). For OH-PBDEs, the binding energies of OH-PBDEs with RAR α (from −8.14 to −9.12 Kcal/M) and RAR γ (from −7.81 to −8.81 Kcal/M) were also considerably higher than for RA (Table 2). These docking results suggest that the PBDEs/OH-PBDEs could not bind with RARs as strongly as RA. The exact binding geometry was different between these compounds (Fig. S1; supplementary materials and methods).

Discussion

Although many studies have reported that PBDEs were capable of disrupting retinoid homeostasis in rodents, birds, and fish, the molecular mechanisms of interactions with the RAR are not fully understood. The present study demonstrates that OH-PBDE exposure could induce RAR transcriptional activity by binding directly with RAR; these effects are possibly related to the structure of the compounds, especially their hydroxylation and bromination. On the contrary, most PBDEs could not directly interact with RARs. To our knowledge, this is the first study to investigate the interactions between PBDEs/OH-PBDEs and zebrafish RARs. Reporter gene assays and molecular docking are valuable approaches for in vitro and in silico investigations to evaluate the RA-disrupting potency of PBDEs/OH-PBDEs and to identify critical structural elements and physicochemical properties of PBDEs/OH-PBDEs related to their hormone activities.

The reporter gene assay results indicated that RAR transcriptional activity could be activated by all tested OH-PBDEs, while most of the tested PBDEs, except for BDE28 and BDE154, had no effect on it. Likewise, several previous studies have reported that PBDEs and their metabolites exhibited different activities on NR-mediated pathways, including thyroid hormone receptor (TR), AR, ER, and GR-mediated pathways. For example, some

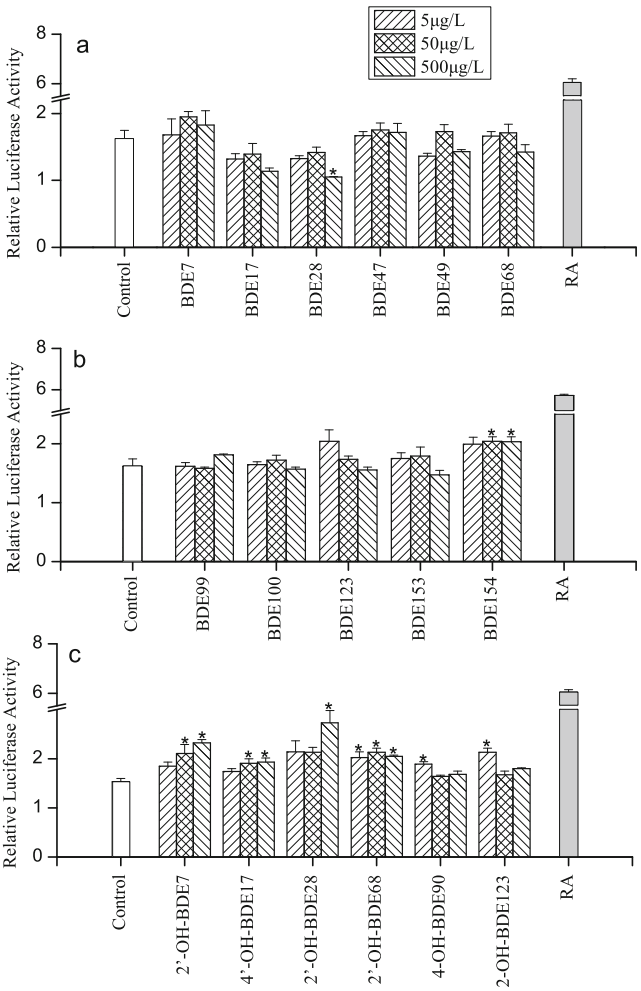

Fig. 2 Luciferase activity induced by PBDE/OH-PBDEs in 293T cells. (**a**) 6 PBDEs; (**b**) 5 PBDEs; (**c**) 6 OH-PBDEs. Data are presented as mean ±SEM of experiments performed in triplicate ($p<0.05$ compared with control)

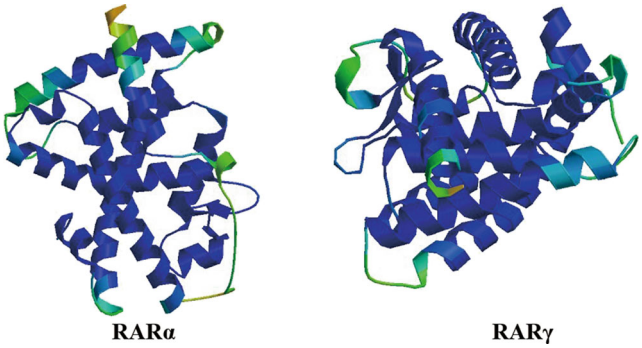

Fig. 3 The ribbon schematic representations of the modeled structures of zebrafish RARα-LBD and RARγ-LBD

studies have demonstrated that OH-PBDEs exhibited TH-disrupting activity through interaction with the TR, while PBDEs did not (Kitamura et al. 2008; Suvorov et al. 2011). Previous studies have also indicated that, compared with PBDEs and MeO-PBDEs, OH-PBDEs exhibited the greatest tendency to act on NR pathways in most cases (Kojima et al. 2009; Li et al. 2010; Ren and Guo 2013). Thus, with respect to the interactions between PBDEs/OH-PBDEs and NRs, our results are consistent with previous findings. However, further studies are needed to investigate the specific ways by which BDE28 and BDE154 can suppress or activate RAR-mediated transcriptional activity.

There are two predominant subtypes of RARs in zebrafish: RAR α and RAR γ. From the molecular docking results, there seems to be a relationship between the binding ability of OH-PBDEs to RAR α and logK_{ow}. As the number of bromines present in OH-PBDEs increased from two to five, their binding affinity with RAR α increased (Table 2). The logK_{ow} increased from 4.84 to 7.33 when the bromination level increased from di- to penta- (Table 2). In fact, there was a positive correlation between the binding ability and logK_{ow} of the OH-PBDEs for RAR α-LBD (R^2=0.92, p<0.05). Similarly, a previous study demonstrated that the binding affinity of 10 OH-PBDEs with TR was correlated to the number of bromines present (R^2=0.96, for TRα-LBD; R^2=0.88, for TRγ-LBD) (Ren et al. 2013), as was that of OH-PCBs (Kitamura et al. 2005). In this study, the degree of bromination of OH-PBDEs was not correlated with RAR γ binding ability (R^2=0.001, p>0.05). Additionally, OH-PBDEs examined in the present study were para- and ortho-substituted, and the position of OH-groups had no observed impact on the interaction of OH-PBDEs with RAR transcription. Conversely, some researchers have reported that the interaction of many OH-PCBs with the ER α/β, AR, and GR depended on the positions of OH-groups and chlorine atoms substituted on their biphenyl structure (Takeuchi et al. 2011).

According to the molecular docking results, the binding energies of PBDEs and OH-PBDEs with RAR-LBD were similar, which indicates that all of these compounds could bind RARs. However, it is particularly interesting that the

Table 2 Molecular volume, logK_{ow} and docking results of 11 PBDEs and 6 OH-PBDEs and RA with zebrafish RARα-LBD and RARγ-LBD

Compound	Volume (cm^3/mol)[a]	LogK_{ow}[b]	RARα-LBD Binding energy (Kcal/M)	RARγ-LBD Binding energy (Kcal/M)
BDE7	633.5	5.23	−8.12	−8.5
BDE17	695.5	6.06	−8.7	−9.07
BDE28	695.5	6.06	−8.48	−8.87
BDE47	757.5	6.89	−8.32	−8.01
BDE49	757.5	6.89	−8.63	−9.04
BDE68	757.5	6.89	−8.88	−8.98
BDE99	819.5	7.72	−8.43	−8.06
BDE100	819.5	7.72	−7.61	−6.66
BDE123	819.5	7.72	−9.49	−8.87
BDE153	881.5	8.54	−8.55	−7.44
BDE154	881.5	8.54	−5.51	−4.49
2′-OH-BDE7	649.5	4.84	−8.14	−8.2
4′-OH-BDE17	711.5	5.67	−8.34	−8.81
2′-OH-BDE28	711.5	5.67	−8.55	−7.81
2′-OH-BDE68	773.5	6.5	−8.57	−7.81
4-OH-BDE90	835.5	7.33	−8.99	−8.20
2-OH-BDE123	835.5	7.33	−9.12	−8.36
RA	1018.5	4.65	−11.28	−11.05

[a] The volume determined by ChemBioDraw

[b] The logK_{ow} values calculated by ChemBioDraw

binding of PBDEs/OH-PBDEs to RARs is not necessary to induce a biological effect. In vitro studies that PBDEs/OH-PBDEs may induce or inhibit RAR transcriptional activity, or may have no effect at all could be examined. With the same degree of bromination, the binding energies of PBDEs and OH-PBDEs were almost the same; the binding energies of RAR α-LBD with BDE7 and 2′-OH-BDE7 were −8.12 and −8.14 Kcal/M, respectively. However, based on the results of the reporter gene assay, 2′-OH-BDE7 directly interacted with RAR α-LBD, but BDE7 did not. These results indicate that although these compounds could bind RARs, the effects of PBDEs/OH-PBDEs on RAR transcriptional activity did not depend on their RAR binding abilities. As a consequence, in the present study, the molecular docking results of PBDEs/OH-PBDEs with zebrafish RAR α-LBD and RAR γ-LBD did not provide sufficient evidence to understand the structural basis of the experimentally observed PBDEs/OH-PBDEs activities in vitro. Further studies including in vivo and in vitro bio-assays and computer modeling are needed to investigate the effects of PBDEs/OH-PBDEs on RAR-mediated pathways and the underlying molecular mechanisms.

Although PBDEs have been shown to disrupt the retinoid homeostasis in rodents, birds, and fish, most of the PBDEs tested in our in vitro study seemed not to directly interact with RAR, suggesting an inability of PBDEs to interfere with RA signaling through RARs. Therefore, the disruption of retinoid homeostasis by PBDE exposure is due to an unknown mechanism. One of the possible mechanisms is related to the metabolism of PBDEs. Several studies have shown that PBDEs can be bio-transformed to OH-PBDEs, which were found to directly interact with RARs in our in vitro study. Since a number of studies have revealed a complex interplay between RA signaling and TH signaling during the development of vertebrates (Bohnsack and Kahana 2013; Essner et al. 1997; Kakizawa et al. 1997), another possible mechanism is related to crosstalk between RA signaling and TH signaling during early development at different levels, including at the level of TR or at the level of the TH transport proteins (such as transthyretin, TTR). Hence, other mechanisms of action of PBDEs/OH-PBDE interference on RA homeostasis and function should be taken into account. Further studies are also necessary to determine whether the mechanisms responsible for receptor-mediated activity vary between different PBDE congeners.

Conclusion

Our study demonstrates that OH-PBDEs could induce RAR transcriptional activity by binding directly with RAR, with the extent of the interaction being dependent on the structure of the compounds (in particular, their hydroxylation and bromination). Most PBDEs could not directly interact with RARs. However, given that the mechanisms of the disruption of retinoid homeostasis by PBDEs are not still fully understood, further molecular toxicology studies are required.

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