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Effects of BDE-99 on AhR receptor in zebrafish larvae

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Abstract: BDE-99 is one of the most abundant PBDEs, which due to its potential persistence and bioaccumulation occurs in aquatic wildlife. Previous studies in mammals have shown that BDE-99 affected development and disrupted certain endocrine functions through signaling pathways mediated by nuclear receptors (NRs). However, fewer studies have investigated the potential of BDE-99 to interact with NRs in aquatic vertebrates, such as fish. In the present study, interactions between BDE-99 and NRs were investigated by use of in silico and in vivo approaches. BDE-99 could dock into ligand binding domain (LBD) of zebrafish aryl hydrocarbon receptor 2 (AhR2) and pregnane X receptor (PXR). BDE-99 had a significant effect on transcriptional profiles of genes associated with AhR or PXR. Based on the developed cytoscape of all zebrafish genes, it was also inferred that AhR and PXR could interact via cross-talk. In addition, both in silico and in vivo results found BDE-99 affected peroxisome proliferator activated receptor alpha (PPARα), glucocorticoid receptor (GR) and thyroid receptor (TR). Collectively, the results presented here demonstrated, for the first time, detailed in silico evidences that BDE-99 could bind to and interact with zebrafish AhR and PXR. These findings can be used to elaborate the molecular mechanism of BDE-99 and guide more objective environmental risk assessments. This article is protected by copyright. All rights reserved

Keywords: Aryl hydrocarbon receptor (AhR), Pregnane X receptor (PXR), Cross-talk, Docking, Molecular dynamic (MD) simulation
INTRODUCTION

Polybrominated biphenyl ethers (PBDEs), a class of organic flame retardants, have been widely used as additives in production of furniture, textiles, building materials and electronic equipment (Talsness 2008). Annual global sales of Penta-BDEs, reported to account for 12% of total PBDE production, were 8,500 and 7,500 tons in 1999 and 2001, respectively (DE Wit 2002; Hites 2004). By 2004 they were phased out of production and use (Stapleton et al. 2009). Due to their continuous leaching from household items (Allen et al. 2008) and also their persistence and potential of bioaccumulation, 2,2’,4,4’,5-Penta-BDE (BDE-99), one of the most abundant Penta-BDEs (Hale et al. 2001), has been detected globally in most environmental compartments, including environmental medium, organisms and humans (Noren and Meironyte 2000; Su et al. 2010; Wang et al. 2011).

Previous studies demonstrated that BDE-99 had adverse effects on the development, neurobehavior and reproduction of rats (Blanco et al. 2013), birds (Eng et al. 2014) and even have adverse effects on humans (Shy et al. 2011). Moreover, many in vitro and in vivo studies of mammals have also been conducted to investigate the endocrine-disrupting effects of BDE-99. For example, BDE-99 could alter expression of thyroid receptor (TR) genes in rat cellular and serum thyroid hormone levels in rat offspring (Blanco et al. 2013; Blanco et al. 2011). It also activated estrogen receptor (ER) in human T47D breast cancer cells (Meerts et al. 2001). BDE-99 was an agonist of the pregnane X receptor (PXR) in mouse based on in vivo and in vitro studies (Pacyniak et al. 2007). Furthermore, BDE-99 had multiple endocrine-disrupting effects...
via nuclear receptors (NRs) in Chinese hamster ovary cells (Kojima et al. 2009). NRs, including aryl hydrocarbon receptor (AhR), ER, androgen receptor (AR), TR, glucocorticoid receptor (GR), peroxisome proliferator activated receptor alpha (PPARα), PXR and mineralocorticoid receptor (MR) can regulate important biological process, such as development, reproduction, and metabolism, by mediating signaling to ligands, such as lipids, and endogenous hormones (Castrillo and Tontonoz 2004; Chinenov et al. 2013). Cytochrome P450 1a1 (Cyp1a1), a biomarker of the activation of AhR-mediated signaling pathway, has been reported to be significantly induced in zebrafish embryos (Usenko et al. 2013) but not be activated in mammalian cells when exposed to BDE-99 (Peters et al. 2006a; Peters et al. 2006b). AhR-mediated response was presumed to be caused by impurities of commercial BDE-99 (Kuiper et al. 2006). Therefore, research was warranted to systematically define NR pathways that could be activated by BDE-99 in fishes, such as through investigations using *in silico* methods.

Thus, to better understand the molecular mechanism of interference of BDE-99 via NRs, both *in vivo* and *in silico* approaches were used to investigate possible effects of BDE-99 on zebrafish (*Danio rerio*) embryos/larvae. To address those questions, three research steps were conducted as follows. First, *in vivo* studies involving changes of gene expression related to several NR pathways were conducted to investigate interference of BDE-99 on endocrine functions of zebrafish. Second, to visualize the effects of BDE-99 on zebrafish regulated by NRs an interaction network demonstrating relationships among genes associated with eight NRs and fold changes in their expression was developed using data generated in step 1. Finally, docking
was used to investigate whether BDE-99 can bind to ligand binding domains (LBD) of NRs, then molecular dynamic (MD) simulations were conducted to further verify the interaction of BDE-99 in zebrafish PXR (z-PXR) as well as zebrafish AhR2 (z-AhR2), which was known to mediate AhR-like effects in zebrafish rather than AhR1a and AhR1b (Prasch et al. 2003; Van Tiem and Di Giulio 2011).

MATERIALS AND METHODS

Materials and reagents

BDE-99 (99.2% purity) was purchased from Accustandard, Inc (New Haven, Connecticut, USA). β-mercaptoethanol was purchased from Amresco LLC (Solon, OH, USA). Stock solutions of BDE-99 were prepared in dimethyl sulfoxide (DMSO, Generay Biotech, Shanghai, China). RNAlater and RNeasy® Mini Kits were obtained from QIAGEN (Hilden, Germany). Omniscript RT Kits were purchased from Thermo (Lithuania, European Union). SYBR® Green Realtime PCR Master Mix Plus Kits were purchased from Toyobo (Tokyo, Japan).

Maintenance of zebrafish and exposure

Adult (7-months old) AB strain zebrafish were maintained in a semi-automatic system, and culture of fish was performed by following OECD Guidelines (OECD 1992). They were fed brine shrimp three times a day. Nylon nets were put in the bottom of tanks to separate embryos from adults. Release and fertilization of eggs were typically initiated within 30 min of turning on the light in the morning. Fertilized embryos were collected and rinsed with embryonic rearing
water. Embryos were then examined under a stereomicroscope and unfertilized and dead individuals were discarded immediately. Healthy embryos were kept in an illuminated incubator at 27±1 °C until 4 h post fertilization (hpf).

In order to determine exposure concentration of BDE-99 on zebrafish at molecular level, lethality and morphological toxicity of BDE-99 was explored during the early life-stages of zebrafish. Embryos (4 hpf) were exposed to 0.04 μM (20 μg/L), 0.4 μM (200 μg/L), or 4 μM (2000 μg/L) until 120 hpf. The results of morphological observations were shown in Fig. S1, abnormal development (e.g., vertebral deformity, pericardial edema, malformed spine) mostly happened in greatest concentrations (4 μM) and some happened at 0.4 μM exposure. Three concentrations including 0.02 μM, 0.1 μM and 0.5 μM were selected for further experiment to explore molecular mechanisms responsible for the adverse effects. Concentrations of DMSO never exceed 0.1% (v/v). Exposures were conducted in 25 mL glass beakers containing 20 mL different concentrations of BDE-99 solution or 0.1% DMSO as the solvent control. Three replicates were tested at each exposed concentration. Twenty embryos at 4 hpf were randomly assigned in each beaker. All containers were kept in an illuminated incubator at 27±1 °C during the exposure experiment. Moreover, unhatched embryos and dead larvae were removed immediately during the experimental period. At 120 hpf, the larvae were sampled and stored in RNAlater solution at -20 °C for further analysis.
**RNA isolation and quantitative real-time polymerase chain reaction (q-RT-PCR)**

Effects of BDE-99 on expression of genes involved in eight receptor signaling pathways were determined by q-RT-PCR as described previously (Liu et al. 2012). Isolation of total RNA was performed using the RNeasy® Mini Kits. The Omniscript RT Kits were used to synthesize cDNA following the manufacturers’ instructions. q-RT-PCR was performed using the SYBR® Green PCR kit under Applied Biosystems Stepone Plus Real-time PCR System. Conditions of the RT-PCR reaction were as follows: initial denaturation step at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Expression of each target gene was normalized to the expression of housekeeping gene *18S* small subunit ribosomal RNA (*18S* rRNA). Changes in genes expression were analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Statistical differences in fold changes between exposure groups and control group were analyzed in GraphPad (GraphPad Software, San Diego, CA, USA) by using one-way analysis of variance (ANOVA) and followed by multiple comparisons Tukey’s Test.

**Structural models preparation**

The three-dimensional (3D) structure of BDE-99 was initially constructed using the sketch molecular module of the Sybyl 7.3 molecular modeling package (Tripos Inc., St. Louis, MO, USA). All hydrogen atoms were added, and compound geometry was subsequently optimized using a Tripos force field with Gasteiger-Hückel charges and minimized using the Powell method with a maximum iteration of 1,000 to reach an energy convergence gradient.
value of 0.001 kcal mol\(^{-1}\)Å (Clark et al. 1989). The minimized structure was used as the initial conformation for molecular docking and MD simulations.

The structural model of the APO form of the zebrafish NRs-LBD was built by homology modeling in the SwissModel workspace (http://swissmodel.expasy.org/workspace/) (Arnold et al. 2006; Kiefer et al. 2009). Amino acid residue sequences of the LBD of z-NRs were downloaded from Uniprot (www.uniprot.org) and the greatest identity of templates were chosen as the modeling templates to construct the z-NRs-LBD structures (Table. S1). The Ramachandran plots were generated in the Structure Analysis and Verification Server (http://services.mbi.ucla.edu/SAVES/) to evaluate the quality of the built z-NRs-LBD.

**Building nuclear receptor pathway**

Eight nuclear receptors were studied. Processes used to build nuclear receptor pathways have been described previously (Liu et al. 2015). Briefly, a biological interaction network of AhR and ER pathways was built using a combination of Cytoscape software v3.1.1 (Cytoscape consortium, San Diego, CA, USA) and the Agilent Literature Search Software (Cline et al. 2007; Doerks et al. 2002), while either SABioscience Gene Network Central (http://www.sabiosciences.com/genenetwork/genenetworkcentral.php) or WikiPathways (http://www.wikipathways.org) (Pico et al. 2008) was used to construct the gene networks of the other six NRs. Additional details are presented in the Supplementary Materials. Only the genes of interested were included in the network pathways. The generated network genes (nodes) were
colored by the Enhanced Graphics application within Cytoscape according to the significant fold changes of genes expression in the respective treatments.

**Docking and MD simulations**

The energy minimized structure of BDE-99 was docked into the APO z-NRs-LBD using the surflex-Dock program of Sybyl 7.3. In molecular docking, an automated model was used to search for a binding pocket. Two factors (threshold and bloat) were set to 0.5 and 1, which can significantly affect the size and shape of binding pockets (Wu et al. 2009). The top Total Score conformations of ligand were selected as the bioactive conformations. Receptors and ligands were merged to be a complex for MD simulation.

MD simulations were carried out using the GROMACS 4 (Hess et al. 2008) package on an International Business Machines (IBM) Blade cluster system. The CHARMM 27 force field was applied to all structural models using GROMACS 4 and SwissParam (http://www.swissparam.ch/) (Zoete et al. 2011). The model was solvated in a box with TIP3P water molecules (Jorgensen et al. 1983), keeping the box boundary at least 10 Å away from any protein atoms. Six sodium ions were subsequently added for charge neutralization. Energy of the system was minimized by the steepest-descent method (Garrett et al. 1988), then the minimized systems were gradually heated from 0 to 300 K at a constant volume for 40 ps with position restraints for ligands. Systems at 300 K were equilibrated for 200 ps with position restraints for ligands and for 1 ns without restraints at 1 bar and 300 K. The MD simulations were then performed in the NPT (constant number of particles, pressure and temperature) ensemble.
periodic boundary conditions. Electrostatic interactions were calculated using the particle mesh Ewald algorithm and van der Waals interactions were accounted for to a cutoff distance of 10 Å. All simulations were carried out for 10 ns using 2 fs time steps, and snapshots for analysis were saved every 2 ps. Data from MD simulations was analyzed in GROMACS 4. The root-mean-square deviation (RMSD), which is the measure of the average distance between the atoms of superimposed proteins, was analyzed by Origin 8 (OriginLab Corp, Northampton, MA, USA).

RESULTS

Transcriptional responses to BDE-99

In the morphological toxicity experiment, mortalities and malformations occurred after exposure of zebrafish to BDE-99 from 4 to 120 hpf. By 24 hpf, development of larvae exposed to 0.4 μM (200 μg/L) or 4 μM BDE-99 (2000 μg/L) was arrested (Fig. S1 B, C, D, and E). Both pericardial edema and deformities of the spine occurred at 120 hpf after exposure to the greatest concentration (4 μM) (Fig. S1 J), and some malformed spines were observed at 96 hpf after exposure to the lesser concentration (0.4 μM) (Fig. S1 G).

In the present study, 54 genes involved in eight receptor-centered gene networks were retrieved according to a previous study (Liu et al. 2015). Furthermore, a q-RT-PCR array was developed to evaluate the effects of BDE-99 on mRNA expression along these constructed gene networks in zebrafish embryos/larvae. Exposure to 0.02, 0.1 or 0.5 μM of BDE-99 resulted in significant changes in expression of genes in several NR signaling pathways, especially AhR2 and PXR (Fig. 1). Gene network of AhR2 was the most affected, since the core receptors of aryl...
hydrocarbon receptor 1b (ahr1b) and aryl hydrocarbon receptor 2 (ahr2) were significantly up-regulated by 2.0 and 2.8-fold following exposure to 0.02 μM of BDE-99, respectively, and were induced by 1.5 and 2.1-fold at 0.5 μM, respectively. Other genes, including aryl hydrocarbon receptor repressor b (ahrrb) and cytochrome P450 1b1 (cyp1b1), were significantly up-regulated compared to control after exposure to either 0.02 or 0.5 μM of BDE-99. The gene aryl hydrocarbon receptor interacting protein (aip) was also significantly up-regulated 1.8-fold at 0.02 μM, and cyp1a1 was significantly up-regulated at the three tested concentrations (Fig. 1 and Table S2). For PXR pathway, pregnane X receptor (pxr), hepatocyte nuclear factor 4, alpha (hnf4a) and cytochrome P450 3a65 (cyp3a65) were increased by 1.5, 1.8 and 2.0-fold, respectively (Fig. 1 and Table S2). For AR, catenin (cadherin-associated protein), beta 1 (ctnnb1), nuclear receptor coactivator 4 (ncoa4), proliferation-associated 2G4, a (pa2g4a) and androgen receptor (ar) genes were significantly up-regulated by 1.7, 1.6, 1.7 and 1.4-fold respectively BDE-99 exposure, while expression of these genes have no significant changes when exposed to two lesser concentrations (Fig. 1 and Table S2). In addition, peroxisome proliferator activated receptor alpha (ppara) and peroxisome proliferator activated receptor gamma (pparg) were significantly induced 1.5 and 2.3-fold at 0.5 μM, respectively (Fig. 1 and Table S2). Expression of glucocorticoid receptor (gr), a receptor gene of GR, and heat shock protein 90kDa alpha family class A member 1 (hsp90aa1) were significantly increased by 1.7 and 1.3-fold when exposed to 0.5 μM (Fig. 1 and Table S2). Among the genes associated with TR, only thyroid receptor alpha (tra) was significantly up-regulated by 1.4-fold at 0.5 μM.
significant alternations in ER and MR core receptor genes pathways were found at any of the
tested concentrations. The gene effects of BDE-99 were not dose-dependent. Changes of 54
genes covering eight NR signaling pathways were presented after exposure of zebrafish to BDE-
99 (Fig. 2). Cyp3a65 that exists in both the AhR- and PXR-signaling pathways was up-regulated
when exposed to 0.5 μM, suggesting that BDE-99 might interact with the endocrine system via
AhR and PXR cross-talk. Meanwhile, the expression of hsp90aa1 could induce the regulation of
AhR and GR at the greatest concentration too (Fig. 2).

Docking and MD simulations between BDE-99 and NRs

High identities indicating good accuracy of the LBDs of z-NRs built by homology
modeling were confirmed through Ramachandran plots (Fig. S2). The surflex-Dock method was
used to automatically search for the binding modes with all parameters set at the default values.

In the present study, BDE-99 successfully docked into all z-NRs-LBD except z-MR (Fig. S3). In
order to predict receptor-ligand interactions more reliably and observe the dynamic behavior of
ligand in the active site of z-AhR2 and z-PXR, which are both the most affected receptors based
on in vivo results, MD simulations were performed. 5 ns was used as the boundary during all
simulations. As for AhR, before 5 ns, the conformation of z-AhR2-LBD changed with time,
which also drove the change in location of the binding site (Fig. 4). After 5 ns, these changes
stopped and the whole complex was stable (Fig. S4). The binding site maintained around
residues Ser138, Ser139, Thr206, Ser209, Pro210, Leu213, Ser214, Phe250, Pro265, Pro266 and
Leu268 all the time (Fig. 4 A). BDE-99 stayed stably in it with no escaping phenomena, which
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laid in the edge of the whole protein and its pocket opened toward the outside of the LBD (Fig. 5 A), with an atom of O forming a strong hydrogen bond with the amino acid residue LEU268. The formation of a hydrogen bond led to strong stability in silico, which indicated that BDE-99 entered the LBD and interacted with the protein (Fig. S5). The relative RMSD fluctuations for the backbone atoms of the BDE-99 molecule and complex were less than 0.2 nm after 5 ns, indicating the stability of ligand and complex had been reached (Figs. S6 and S7). These results supported the fact that BDE-99 bound to the z-AhR2.

In silico results also showed that BDE-99 could bind to the PXR and reached stability quickly according to RMSD (Fig. 5B and Fig. S8). The BDE-99 in the binding pocket is surrounded by Trp4, Val5, Asn7, Thr9, Lys23, His38, Phe39, Leu42, Phe84, Phe87, Trp95, Cys97, Tyr102 (Fig. 5 B). The binding of BDE-99 and PXR were stabilized by forming π-stacking interactions (Tyr102, His38) and a number of hydrophobic interactions (Trp4, Phe39, Leu42, Phe84, Phe87, Trp95, Tyr102). And the RMSD of backbone of the BDE-99 fluctuated less than 0.2 nm after 5 ns (Fig. S9). These results also suggested that BDE-99 can bind to z-PXR stably.
DISCUSSION

Delayed development and malformation observed during in vivo experiments acted as a guide to investigate subtler effects that reveal the toxic mechanisms and potential ecological risk of BDE-99. Therefore, the concentrations around the lowest observed effect concentration (LOEC) were used to investigate transcriptional responses in NR pathways in zebrafish. In vivo investigations revealed that responses of the AhR pathway were the greatest among all the eight measured receptors. Numerous genes relevant to AhR were up-regulated, particularly the core receptor gene ahr2 and biomarker of AhR (cyp1a1), which was consistent with the previous finding that BDE-99 could up-regulate expression of AhR2 target gene cyp1a1 in zebrafish (Usenko et al. 2013). However, some in vitro studies suggested that BDE-99 could bind to AhR2 but could not activate the AhR xenobiotic response element complex and subsequent cyp1a1 transcription processes in several species of mammals (Peters et al. 2006a; Peters et al. 2006b; Peters et al. 2004). Furthermore, other studies indicated that BDE-99 had very small hepatic AhR binding affinities in rat and could not activate rat and human hepatic AhR (Chen et al. 2001; Wahl et al. 2010). Contradiction between the present study and those conducted by others might be a result of the species-specific differences in AhR or the greater sensitivity of early life-stages of zebrafish. However, zebrafish is one of the standard test organisms in toxicology. Research to elucidate mechanisms of BDE-99 can provide basic evidence to comprehensively understand mechanisms of adverse effects caused by BDE-99 on fish. Apart from AhR activity, in vivo results also demonstrated that BDE-99 could activate z-PXR. The results presented here were
consistent with those of previous studies, which showed that BDE-99 could activate PXR of mouse (Pacyniak et al. 2007), supporting that BDE-99 might affect the endocrine system through activation of the PXR signaling pathway. *In silico* analysis including docking and MD were conducted to support the *in vivo* results about AhR and PXR, which were mostly induced. Because information about the crystal structure or the exact binding domain of the z-AhR2 and z-PXR had not been reported, homology modeling was used to construct a molecular model of the LBD of z-AhR2 and PXR. *In silico* results suggested that BDE-99 could bind to and interact with z-AhR and z-PXR, based on the stability of the ligand (BDE-99) and ligand-receptor complex. No escaping phenomenon was observed, indicating that BDE-99 had the potency to activate z-AhR2 and z-PXR through MD simulations. These *in silico* results provided further evidence that BDE-99 can activate the AhR2 and PXR in zebrafish larvae.

Recently, cross-talk between PXR and AhR signaling pathways had been demonstrated in zebrafish (Kubota et al. 2015), which also had been suggested in mammals (Maglich et al. 2002). This cross-talk in zebrafish was considered reciprocal rather than asymmetric, since *ahr2* activation caused up-regulation of *paxr*, cytochrome P450 proteins 2 (*cyp2*) and cytochrome P450 proteins 3 (*cyp3*) genes, and PXR activation up-regulated *ahr2* and *cyp1a1*. Based on this evidence, BDE-99 might affect endocrine systems via cross-talk between PXR and AhR signaling pathways in zebrafish. Also, the cross-talk between AhR and GR signaling pathways was likely another way for BDE-99 to affect endocrine systems, which was consistent with the results of the study of TCDD in human hepatoma cells (Dvorak et al. 2008).
BDE-99 affected the mRNA expression associated with TR and altered the level of thyroid hormone in rats due to its structural similarities with thyroid hormones (Blanco et al. 2013; Blanco et al. 2011). Furthermore, a recent study showed that BDE-99 could alter thyroid hormone concentrations in juvenile Chinook salmon (*Oncorhynchus tshawytscha*) (Arkoosh et al. 2017). These studies were consistent with the *in silico* and *in vivo* results in the present study that BDE-99 could affected zebrafish TR. PPAR plays an important role in lipid homeostasis, inflammation, adipogenesis, reproduction, and carcinogenesis (Abbott 2009). In the present study, BDE-99 increased expression of genes relevant to PPAR in zebrafish, such as *ppara* and *pparg*, and docked into the PPARα successfully. This finding was consistent with results of DE-71 (a PBDEs mixture), which was shown to up-regulate expression of *pparg* at day 8 in 3T3-L1 mouse embryo fibroblast cells (Tung et al. 2014). For GR, previous studies demonstrated that BDE-99 decreased GR activity in adult male rats (Alonso et al. 2010) and had weak antagonistic effects on GR in hamster ovary cells (Kojima et al. 2009). In the present study, BDE-99 could dock into zebrafish GR indicating the likely interaction between BDE-99 and GR. However, GR-relevant genes were up-regulated in zebrafish which was inconsistent with antagonistic effects caused by BDE-99 as observed in adult male rats and hamster ovary cells. This inconsistency in GR-relevant responses between studies might result from differences in responses between mammals and fishes or differences in experimental methodologies used between studies. BDE-99 was demonstrated to dock into ER1 *in silico*, but not into AR. In contrast, expression of genes relevant to AR was altered in zebrafish, but genes relevant to ER1 were not. *In silico* docking
assesses the potential for interaction between receptors and ligands based on structure (Mouchlis et al. 2012), but does not always accurately reflect affinity of the ligand for the receptor \textit{in vivo}. Therefore, the inconsistencies observed between the \textit{in silico} and \textit{in vivo} results in this study for ER1 could be the consequence of the exposure concentrations. Greater concentrations of BDE-99 might induce the expression of ER1-responsive genes in support of the \textit{in silico} docking results. However, the mismatch between \textit{in vivo} and \textit{in silico} results for the AR suggested that BDE-99 was indirectly acting on the AR pathway. Previous studies supported BDE-99 acting on AR-responses based on an increase in androstenedione and testosterone secretion from ovarian follicles (Gregoraszczuk et al. 2008; Karpeta et al. 2011). Therefore, alteration in AR-responsive genes might represent compensatory responses of the AR-pathway in response to other mechanisms of BDE-99. However, compensatory responses of the endocrine system were complex which made it necessary to consider multiple nuclear receptor pathways comprising the endocrine system in order to identify the underlying mechanism(s). Therefore, additional investigations were needed to clarify toxicological mechanisms of BDE-99 mediated through complex interactions between the AR, ER1, and other endocrine pathways. Finally, both \textit{in silico} and \textit{in vivo} results demonstrated that BDE-99 had no effect on MR.

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CONCLUSIONS

The present study is the first to elucidate molecular aspects of the endocrine disrupting effects induced by BDE-99 in zebrafish by both in silico and in vivo approaches. Interactions of BDE-99 with the z-AhR2 and z-PXR were characterized by use of docking and molecular dynamic simulations. Experimental results regarding the molecular analysis of response patterns of key genes along NR pathways further verified that BDE-99 was an agonist of AhR as well as PXR of zebrafish. Our findings provide insight into the interaction of BDE-99 with steroid hormone receptor pathways, which may offer novel clues for the molecular mechanism of endocrine disruption and developmental toxicity in aquatic vertebrates.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.xxxx.
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The numerical calculations in this paper were performed on the IBM Blade cluster system in the High Performance Computing Center (HPCC) of Nanjing University.

Data availability—Data are available in the Supplementary Data files.

This article includes online-only Supplemental Data.

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Figure 1. The volcano plot of gene expression profiles in each NR pathway. Genes involved in different exposure concentrations and NR pathways were given with different shapes and colors (see legend).

Figure 2. Interaction network shows the relationship among genes associated with eight receptor pathways in zebrafish and the expression changes of these genes after exposure to 0.02, 0.1 and 0.5 μM of BDE-99, respectively. Nodes represent single genes and edges indicate either protein-protein or protein-DNA interactions. The red color represents significant up-regulation and green color represents significant down-regulation of genes.

Figure 3. Changes in the location of binding site were driven by the conformation changes of BDE-99-AhR2 complex. Blue and red compounds represent BDE-99 at 0 ns and 5 ns, respectively. The picture was generated and captured by PyMol (Ver.0.99, open source).

Figure 4. Binding pocket of zebrafish (A) AhR2 and (B) PXR with several key residues. Red compound represents BDE-99. The picture was generated and captured by PyMol (Ver.0.99, open source).

Figure 5. BDE-99 and binding pocket of (A) AhR2 and (B) PXR. The picture was generated and captured by PyMol (Ver.0.99, open source).

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Figure 1

-log10 p value vs Log2 (Fold change)

Dots represent different concentrations of various receptors:
- AhR2 (0.02 μM, 0.1 μM, and 0.5 μM)
- ER1 (0.02 μM, 0.1 μM, and 0.5 μM)
- PPARα (0.02 μM, 0.1 μM, and 0.5 μM)
- PXR (0.02 μM, 0.1 μM, and 0.5 μM)
- MR (0.02 μM, 0.1 μM, and 0.5 μM)
- TR (0.02 μM, 0.1 μM, and 0.5 μM)
- GR (0.02 μM, 0.1 μM, and 0.5 μM)
- AR (0.02 μM, 0.1 μM, and 0.5 μM)