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Integrated In Silico and In Vivo Approaches to Investigate Effects of BDE-99 Mediated by the Nuclear Receptors on Developing Zebrafish

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Abstract: One of the most abundant polybrominated diphenyl ethers (PBDEs) is 2,2′,4,4′,5-pentabromodiphenyl ether (BDE-99), which persists and potentially bioaccumulates in aquatic wildlife. Previous studies in mammals have shown that BDE-99 affects development and disrupts certain endocrine functions through signaling pathways mediated by nuclear receptors. However, fewer studies have investigated the potential of BDE-99 to interact with nuclear receptors in aquatic vertebrates such as fish. In the present study, interactions between BDE-99 and nuclear receptors were investigated by in silico and in vivo approaches. This PBDE was able to dock into the ligand-binding domain of zebrafish aryl hydrocarbon receptor 2 (AhR2) and pregnane X receptor (PXR). It had a significant effect on the 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 the in silico and in vivo approaches found that BDE-99 affected peroxisome proliferator–activated receptor alpha (PPARα), glucocorticoid receptor, and thyroid receptor. Collectively, our results demonstrate for the first time detailed in silico evidence that BDE-99 can 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. *Environ Toxicol Chem* 2018;37:780–787. © 2017 SETAC

Keywords: Aryl hydrocarbon receptor; Pregnane X receptor; Cross-talk; Docking; Molecular dynamic simulation

INTRODUCTION

Polybrominated biphenyl ethers (PBDEs), a class of organic flame retardants, have been widely used as additives in the 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 8500 and 7500 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). Because of their continuous leaching from household items (Allen et al. 2008) and their persistence and bioaccumulation potential, 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 media, organisms, and humans (Noren and Meironyte 2000; Su et al. 2010; Wang et al. 2011).

Previous studies have demonstrated that BDE-99 has adverse effects on the development, neurobehavior, and reproduction of rats (Blanco et al. 2013), birds (Eng et al. 2014), and 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 was able to alter expression of thyroid receptor genes in rat cellular and serum thyroid hormone levels in rat offspring (Blanco et al. 2011, 2013). It also activated estrogen receptor (ER) in human T47D breast cancer cells (Meerts et al. 2001). Based on in vivo and in vitro studies, BDE-99 was shown to be an agonist of the pregnane X receptor (PXR) in mouse (Pacyniak et al. 2007). Furthermore, BDE-99 had multiple endocrine-disrupting effects via nuclear receptors in Chinese hamster ovary cells

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(Kojima et al. 2009). The nuclear receptors, including aryl hydrocarbon receptor (AhR), ER, androgen receptor (AR), thyroid receptor, glucocorticoid receptor, peroxisome proliferator–activated receptor alpha (PPARα), PXR, and mineralocorticoid receptor (MR) regulate important biological processes (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 the AhR-mediated signaling pathway, has been reported to be significantly induced in zebrafish embryos (Usenko et al. 2013) but not to be activated in mammalian cells when exposed to BDE-99 (Peters et al. 2006a and b). The AhR-mediated response was presumed to be the result of impurities of commercial BDE-99 (Kuiper et al. 2006). Therefore, research was warranted to systematically define nuclear receptor pathways that could be activated by BDE-99 in fish, such as through investigations using in silico methods.

Thus, to better understand the molecular mechanism of interference of BDE-99 via nuclear receptors, both in vivo and in silico approaches were used to investigate possible effects of BDE-99 on zebrafish (*Danio rerio*) embryos and larvae. First, in vivo studies involving changes in gene expression related to several nuclear receptor pathways were conducted to investigate the interference of BDE-99 with endocrine functions of zebrafish. Second, to visualize the effects of BDE-99 on zebrafish regulated by nuclear receptors, an interaction network demonstrating relationships among genes associated with 8 nuclear receptors 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 of nuclear receptors, and then molecular dynamic simulations were conducted to further verify the interaction of BDE-99 in zebrafish-PXR as well as zebrafish-AhR2, which is 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

The BDE-99 (99.2% purity) was purchased from Accustandard. β-Mercaptoethanol was purchased from Amresco. Stock solutions of BDE-99 were prepared in dimethyl sulfoxide (DMSO; Generay Biotech). RNAlater and RNeasy® Mini kits were obtained from Qiagen. Omniscript RT kits were purchased from Thermo. SYBR® Green Realtime PCR Master Mix Plus kits were purchased from Toyobo.

Maintenance of zebrafish and exposure

Adult (7-mo-old) AB strain zebrafish were maintained in a semiautomatic system, and fish were cultured following guidelines of the Organisation for Economic Co-operation and Development (1992). They were fed brine shrimp 3 times a day. Nylon nets were put in the bottom of the 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).

To determine the effects of exposure concentrations of BDE-99 at a molecular level, the 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 the morphological observations are shown in the Supplemental Data, Figure S1; abnormal developments (e.g., vertebral deformity, pericardial edema, malformed spine) mostly occurred at the greatest concentration (4 μM), with some occurring at 0.4 μM exposure. Three concentrations (0.02, 0.1, and 0.5 μM) were selected for further experiment to explore the molecular mechanisms responsible for the adverse effects. Concentrations of DMSO never exceeded 0.1% (v/v). Exposures were conducted in 25-mL glass beakers containing 20 mL of different concentrations of BDE-99 solution or 0.1% DMSO as the solvent control. Three replicates were tested at each concentration. Twenty embryos at 4 hpf were randomly assigned to each beaker. All containers were kept in an illuminated incubator at 27 ± 1 °C during the exposure experiment, and 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

Effects of BDE-99 on the expression of genes involved in 8 receptor signaling pathways were determined by quantitative real-time polymerase chain reaction (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 complementary DNA (cDNA) following the manufacturers' instructions. The q-RT-PCR was performed using the SYBR® Green PCR kit under the 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) by using one-way analysis of variance followed by multiple comparisons Tukey's test.

Structural models preparation

The 3-dimensional structure of BDE-99 was initially constructed using the sketch molecular module of the Sybyl 7.3 molecular modeling package (Tripos). 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 1000 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 molecular dynamic simulations.

The structural model of the APO form of the zebrafish nuclear receptors–ligand-binding domain was built by homology modeling in the SwissModel workspace (Arnold et al. 2006; Kiefer et al. 2009). Amino acid residue sequences of the ligand-binding domain of zebrafish-nuclear receptors were downloaded from Uniprot, and the greatest identities of templates were chosen as the modeling templates to construct the zebrafish-nuclear receptors–ligand-binding domain structures (Supplemental Data, Table S1). The Ramachandran plots were generated in the Structure Analysis and Verification Server to evaluate the quality of the built zebrafish-nuclear receptors–ligand-binding domain.

Building the 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 Ver 3.1.1 and the Agilent Literature Search Software (Cline et al. 2007; Doerks et al. 2002), whereas either SABioscience Gene Network Central or WikiPathways (Pico et al. 2008) was used to construct the gene networks of the other 6 nuclear receptors. Additional details are presented in the Supplemental Data. Only the genes of interest 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 gene expression in the respective treatments.

Docking and molecular dynamic simulations

The energy minimized structure of BDE-99 was docked into the APO zebrafish-nuclear receptors–ligand-binding domain 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 the 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 molecular dynamic simulation.

The molecular dynamic simulations were carried out using the GROMACS 4 (Hess et al. 2008) package on an IBM Blade cluster system. The CHARMM 27 force field was applied to all structural models using GROMACS 4 and SwissParam (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), and 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 molecular dynamic simulations were then performed in the NPT (constant number of particles, pressure, and temperature) ensemble with 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 molecular dynamic simulations were 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).

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 (Supplemental Data, Figure S1B–E). Both pericardial edema and deformities of the spine occurred at 120 hpf after exposure to the greatest concentration (4 μM; Supplemental Data, Figure S1J), and some malformed spines were observed at 96 hpf after exposure to the lesser concentration (0.4 μM; Supplemental Data, Figure S1G).

In the present study, 54 genes involved in 8 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 and larvae. Exposure to 0.02, 0.1, or 0.5 μM of BDE-99 resulted in significant changes in expression of genes in several nuclear receptor signaling pathways, especially AhR2 and PXR (Figure 1). The gene network of AhR2 was the most affected, because 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, respectively, following exposure to 0.02 μM of BDE-99, and were induced by 1.5- and 2.1-fold, respectively, at 0.5 μM. Other genes, including aryl hydrocarbon receptor repressor b (*ahrrb*) and cytochrome P450 1b1 (*cyp1b1*), were significantly up-regulated compared with 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 by 1.8-fold at 0.02 μM, and *cyp1a1* was significantly up-regulated at the 3 tested concentrations (Figure 1 and Supplemental Data, Table S2). For the 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 (Figure 1 and Supplemental Data, Table S2). For AR, catenin (cadherin-associated protein), beta 1 (*ctnnb1*), nuclear receptor

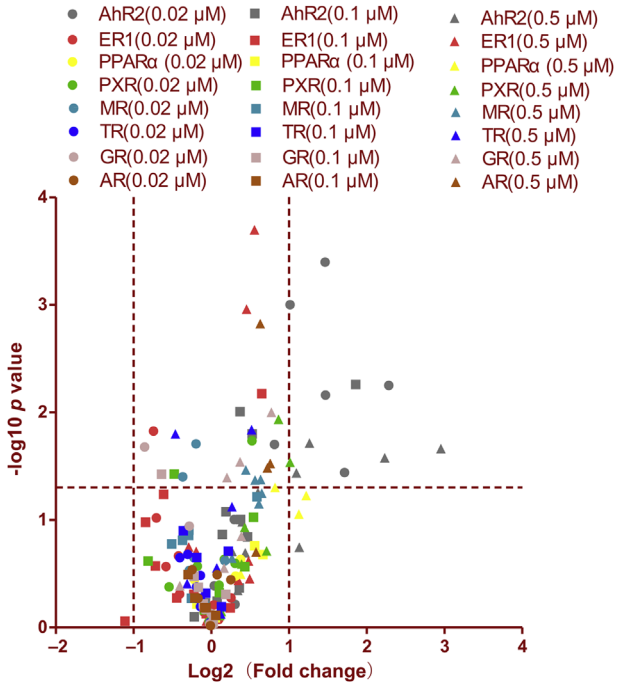

FIGURE 1: The volcano plot of gene expression profiles in each nuclear receptor pathway. Genes involved in different exposure concentrations and nuclear receptor pathways have different shapes and colors (see the key). AhR = aryl hydrocarbon receptor; ER = estrogen receptor; PPARα = peroxisome proliferator activated receptor alpha; PXR = pregnane X receptor; MR = mineralocorticoid receptor; TR = thyroid receptor; GR = glucocorticoid receptor; AR = androgen 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, whereas expression of these genes was not significantly changed after exposure to 2 lesser concentrations (Figure 1 and Supplemental Data, Table S2). In addition, peroxisome proliferator activated receptor alpha (*ppara*) and peroxisome proliferator activated receptor gamma (*pparg*) were significantly induced by 1.5- and 2.3-fold, respectively, at 0.5 μM (Figure 1 and Supplemental Data, Table S2). Expression of glucocorticoid receptor (*gr*), a receptor gene of glucocorticoid, and heat shock protein 90kDa alpha family class A member 1 (*hsp90aa1*) were significantly increased by 1.7- and 1.3-fold after exposure to 0.5 μM (Figure 1 and Supplemental Data, Table S2). Among the genes associated with the thyroid receptor, only thyroid receptor alpha (*tra*) was significantly up-regulated by 1.4-fold at 0.5 μM. No significant alterations 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 in 54 genes covering 8 nuclear receptor signaling pathways were seen after exposure of zebrafish to BDE-99 (Figure 2). The *Cyp3a65* gene, which exists in both the AhR- and PXR-signaling pathways was up-regulated after exposure to 0.5 μM, suggesting that BDE-99 might interact with the endocrine system via AhR and PXR cross-talk. Meanwhile, *hsp90aa1* expression also induced regulation of the AhR and glucocorticoid receptor at the highest concentration (Figure 2).

Docking and molecular dynamic simulations between BDE-99 and nuclear receptors

High identities indicating good accuracy of the ligand-binding domains of zebrafish-nuclear receptors built by homology modeling were confirmed through Ramachandran plots (Supplemental Data, Figure S2). The surflex-Dock method was used to automatically search for the binding modes with all parameters set at the default values. Except for zebrafish-MR, BDE-99 successfully docked into all zebrafish-nuclear receptors–ligand-binding domain (Supplemental Data, Figure S3). To predict receptor–ligand interactions more reliably and observe the dynamic behavior of ligand in the active sites of zebrafish-

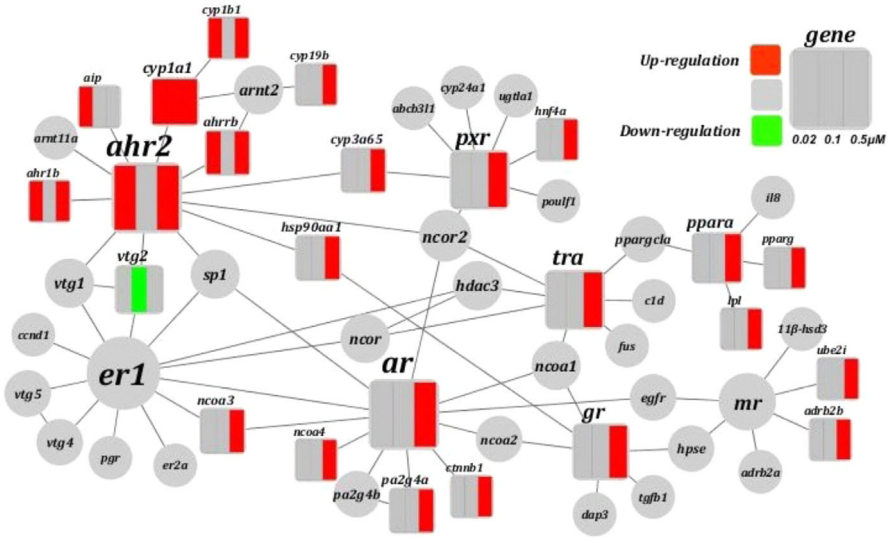

FIGURE 2: Interaction network showing the relationship among genes associated with 8 receptor pathways in zebrafish and the expression changes of these genes after exposure to 0.02, 0.1, and 0.5 μM of BDE-99, respectively. Nodes represent single genes, and edges indicate either protein–protein or protein–DNA interactions. The red color represents significant up-regulation and the green color represents significant down-regulation of genes. All gene names are shown in Supplemental Data (Table S2).

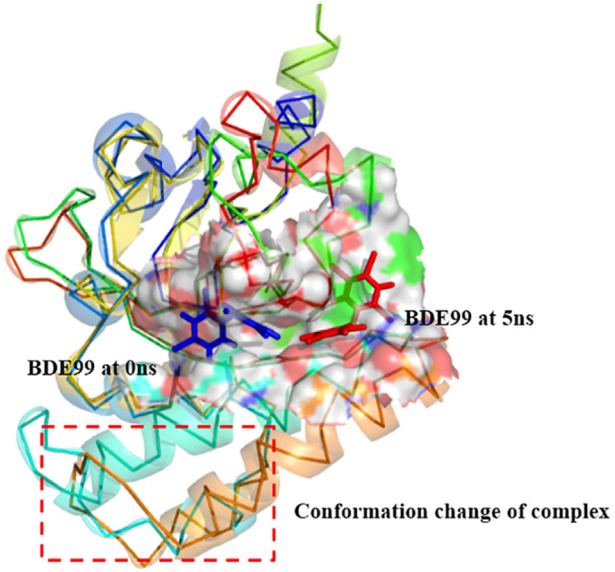

FIGURE 3: Changes in the location of binding sites were driven by the conformation changes of the BDE-99–aryl hydrocarbon receptor 2 complex. Blue and red compounds represent BDE-99 at 0 ns and 5 ns, respectively. The picture was generated and captured by PyMol (Ver 0.99, open source).

AhR2 and zebrafish-PXR, which are the 2 most affected receptors based on in vivo results, molecular dynamic simulations were performed. Five nanoseconds were used as the boundary during all simulations. As for AhR, before 5 ns, the conformation of zebrafish-AhR2–ligand-binding domain changed with time, which also drove the change in location of the binding site (Figure 3). After 5 ns, these changes stopped and the whole complex was stable (Supplemental Data, Figure S4). The binding site remained for the whole time around residues Ser138, Ser139, Thr206, Ser209, Pro210, Leu213, Ser214, Phe250, Pro265, Pro266, and Leu268 (Figure 4A). The BDE-99 stayed stably in the binding site with no escaping residue. The residue lay at the edge of the whole protein and its pocket opened toward the outside of the ligand-binding domain (Figure 5A), 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 ligand-binding domain and interacted with the protein (Supplemental Data, Figure 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 that stability of ligand and complex had been reached (Supplemental Data, Figures S6 and S7). These results support the fact that BDE-99 bound to the zebrafish-AhR2.

In silico results also showed that BDE-99 bound to the PXR and reached stability quickly according to the RMSD (Figure 5B and Supplemental Data, Figure S8). The BDE-99 in the binding pocket was surrounded by Trp4, Val5, Asn7, Thr9, Lys23, His38, Phe39, Leu42, Phe84, Phe87, Trp95, Cys97, and Tyr102 (Figure 5B). The binding of BDE-99 and PXR was stabilized by forming π-stacking interactions (Tyr102, His38) and a number of hydrophobic interactions (Trp4, Phe39, Leu42, Phe84, Phe87, Trp95, Tyr102). Also, the RMSD of the backbone of the BDE-99 fluctuated less than 0.2 nm after 5 ns (Supplemental Data, Figure S9). These results also suggested that BDE-99 can bind to zebrafish-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 at approximately the lowest-observed-effect concentration were used to investigate transcriptional responses in nuclear receptor pathways in zebrafish. In vivo investigations revealed that responses of the AhR pathway were the greatest among all 8 receptors measured. Numerous genes relevant to AhR were up-regulated, particularly the core receptor gene *ahr2* and the biomarker of AhR (*cyp1a1*), which was consistent with the previous finding that BDE-99 was able to up-regulate expression of the 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, 2004 and b). Furthermore, other studies indicated that BDE-99 had very small hepatic AhR binding affinities in rat and could not activate rat and human

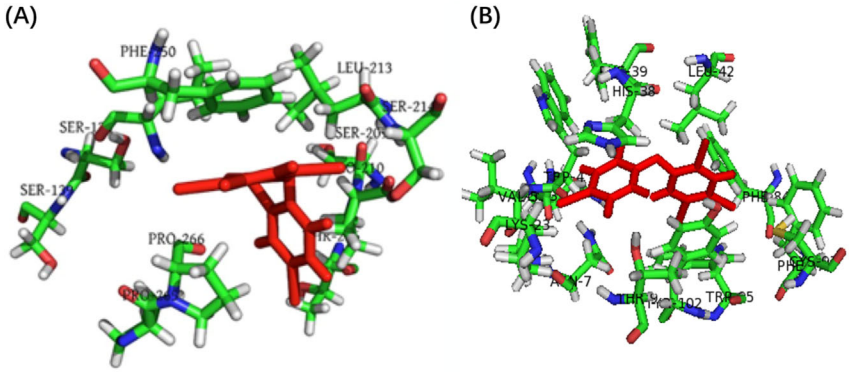

FIGURE 4: The binding pocket of zebrafish (A) aryl hydrocarbon receptor 2 and (B) pregnane X receptor with several key residues. Red compound represents BDE-99. The picture was generated and captured by PyMol (Ver 0.99, open source).

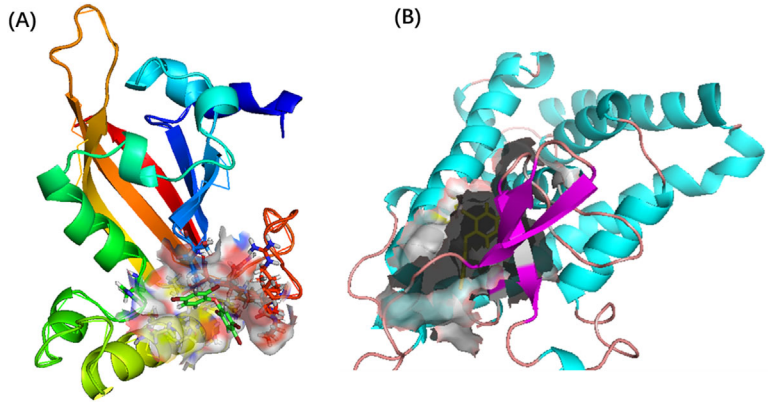

FIGURE 5: The BDE-99 and binding pocket of (**A**) aryl hydrocarbon receptor 2 and (**B**) pregnane X receptor. The picture was generated and captured by PyMol (Ver 0.99, open source).

hepatic AhR (Chen et al. 2001; Wahl et al. 2010). The contradictions 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 that will allow us to comprehensively understand mechanisms of adverse effects on fish as a result of BDE-99. Apart from AhR activity, in vivo results also demonstrated that BDE-99 could activate zebrafish-PXR. Our results were consistent with those of previous studies, which showed that BDE-99 could activate PXR of mouse (Pacyniak et al. 2007), supporting the hypothesis that BDE-99 might affect the endocrine system through activation of the PXR signaling pathway. In silico analysis including docking and molecular dynamic were conducted to support the in vivo results on AhR and PXR, which were mostly induced. Because information about the crystal structure or the exact binding domain of the zebrafish-AhR2 and zebrafish-PXR had not been reported, homology modeling was used to construct a molecular model of the ligand-binding domain of zebrafish-AhR2 and PXR. In silico results suggested that BDE-99 could bind to and interact with zebrafish-AhR and zebrafish-PXR, based on the stability of the ligand (BDE-99) and ligand–receptor complex. No escaping phenomena were observed, indicating that BDE-99 had the ability to activate zebrafish-AhR2 and zebrafish-PXR through molecular dynamic simulations. These in silico results provided further evidence that BDE-99 can activate the AhR2 and PXR in zebrafish larvae.

Cross-talk between PXR and AhR signaling pathways was recently demonstrated in zebrafish (Kubota et al. 2015), and was also suggested in mammals (Maglich et al. 2002). This cross-talk in zebrafish was considered reciprocal rather than asymmetric, because *ahr2* activation caused up-regulation of *pxr*, cytochrome P450 proteins 2 (*cyp2*), and cytochrome P450 proteins 3 (*cyp3*) genes, and PXR activation up-regulated *ahr2* and *cyp1a1*. Based on this evidence, it is possible that BDE-99 affects endocrine systems via cross-talk between PXR and AhR signaling pathways in zebrafish. Also, the cross-talk between AhR and glucocorticoid receptor signaling pathways was likely another way for BDE-99 to affect endocrine systems, which was consistent with the results of the study of tetrachlorodibenzodioxin in human hepatoma cells (Dvorak et al. 2008).

Because of its structural similarities to thyroid hormones, BDE-99 affected the mRNA expression associated with the thyroid receptor and altered the level of thyroid hormone in rats (Blanco et al. 2013, 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 showing that BDE-99 could affect the zebrafish thyroid receptor. The transcription factor 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 successfully docked into PPARα. This finding was consistent with results for DE-71 (a mixture of PBDEs), which was shown to up-regulate expression of *pparg* at day 8 in 3T3-L1 mouse embryo fibroblast cells (Tung et al. 2014). For the glucocorticoid receptor, previous studies demonstrated that BDE-99 decreased glucocorticoid receptor activity in adult male rats (Alonso et al. 2010) and had weak antagonistic effects on the glucocorticoid receptor in hamster ovary cells (Kojima et al. 2009). In the present study, BDE-99 docked into the zebrafish glucocorticoid receptor, indicating a likely interaction between BDE-99 and the glucocorticoid receptor. However, glucocorticoid receptor-relevant genes were up-regulated in zebrafish, which was inconsistent with antagonistic effects because of BDE-99 as observed in adult male rats and hamster ovary cells. This inconsistency in glucocorticoid receptor-relevant responses between studies might result from differences in responses between mammals and fishes or differences in experimental methodologies used between studies. It was demonstrated that BDE-99 docked 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 in vivo. Therefore, the inconsistencies observed between the in silico and in vivo results in the present study for ER1 could be the consequence of the exposure concentrations. Greater concentrations of BDE-99 might induce expression of ER1-responsive genes in support of the in silico docking results. However, the mismatch between in vivo and in silico results for AR suggested that BDE-99 was indirectly acting on the AR pathway. Previous studies supported the possibility that BDE-99 acts 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 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 in silico and in vivo results demonstrated that BDE-99 had no effect on MR.

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 zebrafish-AhR2 and zebrafish-PXR were characterized by using docking and molecular dynamic simulations. Experimental results regarding the molecular analysis of response patterns of key genes along nuclear receptor pathways further verified that BDE-99 was an agonist of AhR as well as PXR of zebrafish. Our findings provide insights into the interaction of BDE-99 with steroid hormone receptor pathways, which may offer novel clues toward 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.4000.

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

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Supporting Information

Integrated *in silico* and *in vivo* approaches to investigate effects of BDE-99 mediated by the nuclear receptors on developing zebrafish

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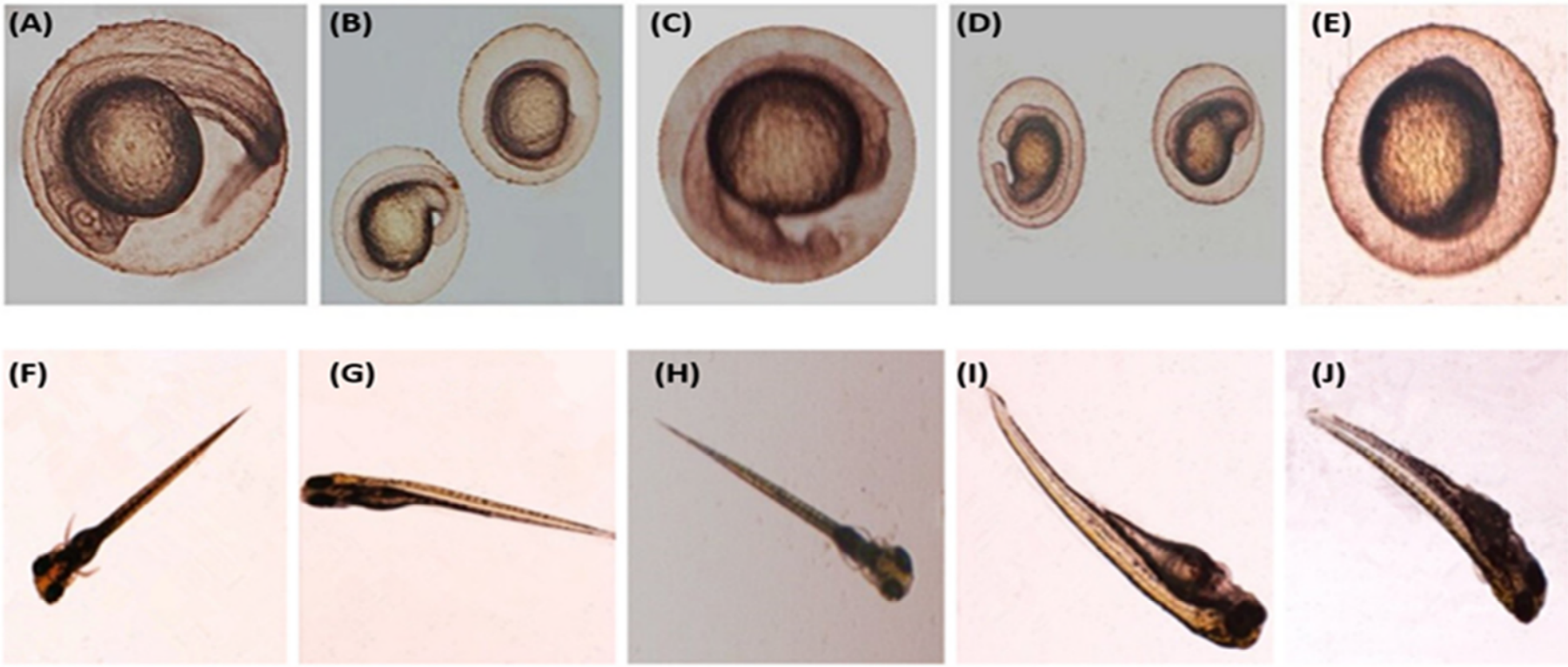
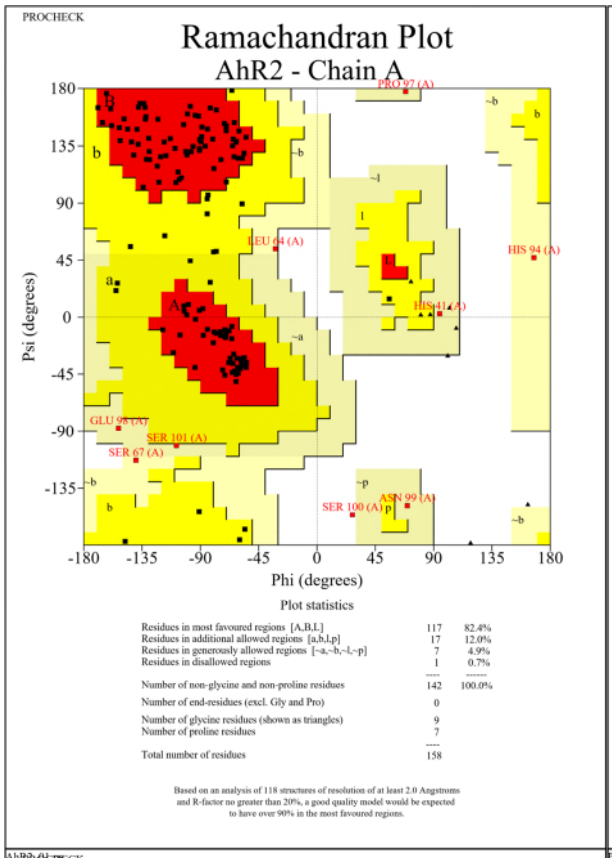
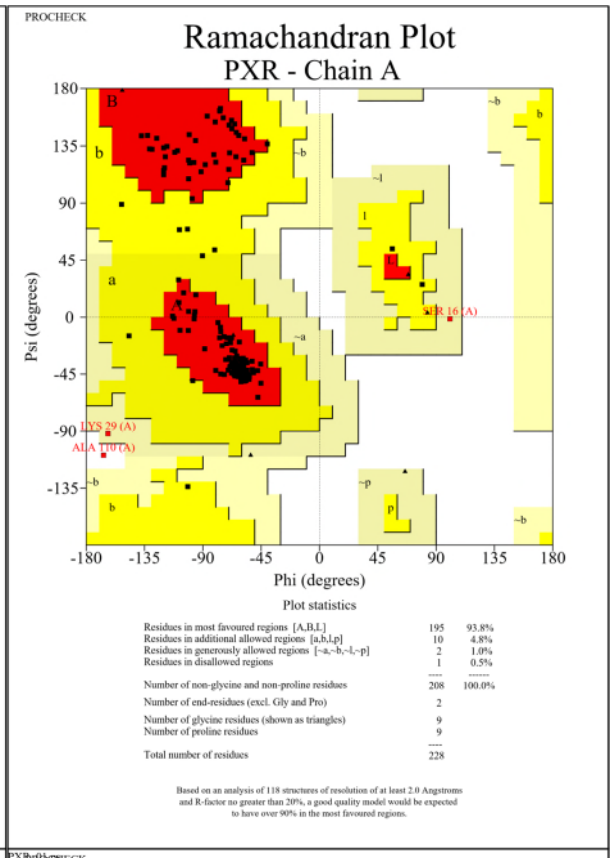
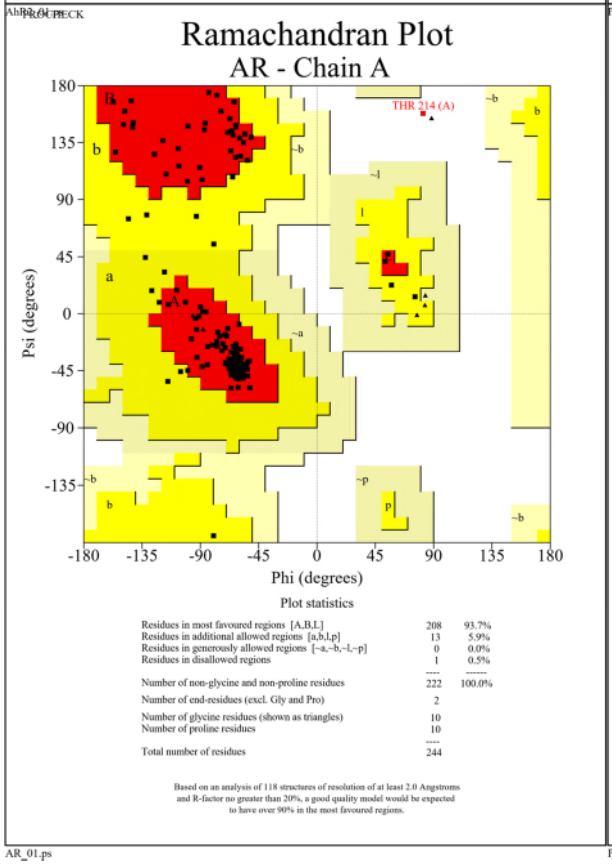


Fig. S1. Photomicrographs demonstrating changes in morphology at several stages of development of zebrafish embryo exposed to BDE-99. (A) Normal developed embryo (24 hpf); (B), (C) and (D): Delayed development embryo exposed to 0.4 μM (200 μg/L) BDE-99 (24 hpf); (E): Abnormal embryo exposed to 4 μM (2000 μg/L) BDE-99 (24 hpf); (F): Normal developed zebrafish larvae (96 hpf); (G): Malformed spine after exposure to 0. 4 μM BDE-99 (96 hpf); (H): Normal developed zebrafish larvae (120 hpf); (I): Pericardial edema after being exposed to 0. 4 μM BDE-99 (120 hpf); (J): Pericardial edema and malformed spine after exposed to 4 μM BDE-99 (120 hpf).







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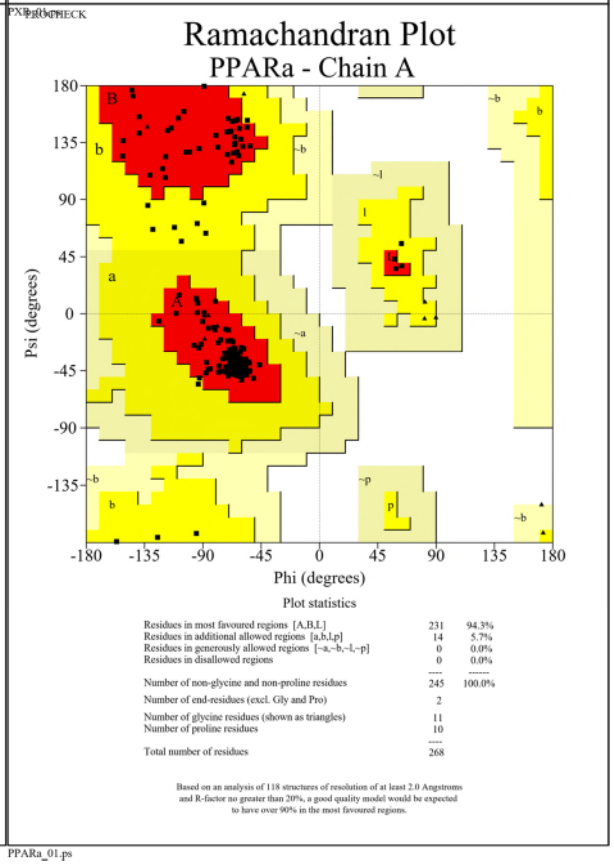

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