Toxicology and Applied Pharmacology 305 (2016) 203–215

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

BDE-99, but not BDE-47, is a transient aryl hydrocarbon receptor agonist in zebrafish liver cells

Jie Yang, Jinyong Zhu [1], King Ming Chan *

School of Life Sciences, The Chinese University of Hong Kong, Sha Tin, N.T., Hong Kong Special Administrative Region

ARTICLE INFO

Article history:
Received 19 January 2016
Revised 19 June 2016
Accepted 20 June 2016
Available online 23 June 2016

Key words:
Ahr activation
CYP1A
BDE-99
Zebrafish
Reporter-gene assay

ABSTRACT

Polybrominated diphenyl ethers (PBDEs) are endocrine-disrupting chemicals that affect the environment and the health of humans and wildlife. In this study, the zebrafish liver (ZFL) cell line was used *in vitro* to investigate two major PBDE contaminants: 2, 2′, 4, 4′, 5-pentabromodiphenyl ether (BDE-99) and 2, 2′, 4, 4′-tetrabromodiphenyl ether (BDE-47). BDE-99 was found to significantly induce cytochrome P450 (*CYP1A*), uridine diphosphate glucuronosyl transferase 1 family a, b (*ugt1ab*), 7-ethoxyresorufin-*O*-deethylase activity and an aryl hydrocarbon receptor (Ahr) dependent xenobiotic response element luciferase reporter system, confirming the Ahr-mediated activation of CYP1A by BDE-99. The time-course effect indicated that the role of BDE-99 in Ahr-mediated signaling is likely to be transient and highly dependent on the ability of BDE-99 to induce *CYP1A* and *ugt1ab*, and presumably its metabolism. BDE-99 also exhibited a significant dose-response effect on a developed zebrafish pregnane X receptor luciferase reporter gene system. However, the other abundant contaminant under study, BDE-47, did not exhibit the above effects. Together, these results indicated that the molecular mechanism of PBDEs induced in ZFL cells is a chemically specific process that differs between members of the PBDE family. CYP1A induction derived by BDE-99 warrants further risk assessment as the humans, wildlife and environment are exposed to a complex mixture including dioxin-like compounds and carcinogenic compounds.

1. Introduction

PBDEs are used as flame retardants in a wide variety of products, such as plastics, furniture, upholstery, electrical equipment, electronic devices, textiles and other household products (ATSDR, 2004; EPA, 2009). They comprise 209 congeners with different numbers or positions of bromine atoms, and share a diphenyl-ether structure. Three types of commercial PBDE mixture, namely pentabromodiphenyl ether (pentaBDE), octabromodiphenyl ether (octaBDE) and decabromodiphenyl ether (decaBDE), have been extensively used in industrial contexts. In the United States, the production of pentaBDE and octaBDE has been voluntarily ceased in 2004, and decaBDE is also phased out in 2009 due to their potential health risks (EPA, 2009). PBDE contamination is an increasing issue, as PBDEs accumulate in the environment and wildlife until they degrade or are eliminated, however, the dismantling of electronic wastes persistently releases huge amount of PBDEs into the environment. Serious contamination has been detected in electronic-waste recycling sites in developing countries (Leung et al., 2006; Bi et al., 2007). The most abundant contaminants are 2, 2′, 4, 4′-tetrabromodiphenyl ether (BDE-47) and 2, 2′, 4, 4′, 5-pentabromodiphenyl ether (BDE-99). Dietary food and indoor dust are major sources of human exposure to BDE-47 and BDE-99 (Wu et al., 2007). The high concentration of PBDEs in samples taken from Hong Kong residents has been reported to be associated with their consumption of marine and freshwater fish (Cheung et al., 2008). Another issue of concern is that PBDE levels are higher in children than adults. Researchers have found that high PBDE levels in children are related to decreased fine-manipulation abilities and reduced attention span (Roze et al., 2009; Herbstman et al., 2010). Lower brominated congeners are especially persistent in human bodies, with half-lives ranging from 2 to 12 years (Geyer et al., 2004).

PBDEs are defined as endocrine-disrupting chemicals (EDCs) due to their structural similarity to endocrine hormones, such as thyroid hormones (THs). Using human, rodent, fish and other models both *in vivo* and *in vitro*, numerous researchers have shown that PBDEs have toxic effects on the hypothalamus-pituitary-thyroid axis, TH metabolism (Marchesini et al., 2008; Butt et al., 2011; Li et al., 2010; Kojima et al., 2009; Chan and Chan, 2012; Yang and Chan, 2015), neurodevelopment (Costa and Giordano, 2007; Costa et al., 2014) and energy balance (Hoppe and Carey, 2007). These effects are typically observed in patients with obesity and metabolic syndromes (Fernandez et al., 2007).

* Corresponding author.
E-mail address: kingchan@cuhk.edu.hk (K.M. Chan).
[1] Biomedical Research Institute, Shenzhen Peking University - The Hong Kong University of Science and Technology Medical Center, Shenzhen, Guangdong, China.

http://dx.doi.org/10.1016/j.taap.2016.06.023
0041-008X/

However, the mechanisms underlying the toxic effects of PBDEs remain unclear, as the results obtained are chemical- and species-specific, and sometimes even vary within the same chemical or species.

The structural similarity with another class of compounds, namely polychlorinated biphenyls (PCBs), also raised people's concern about their possibility to agonize aryl hydrocarbon receptor (AHR)-mediated cytochrome P450 1A (CYP1A) induction (ATSDR, 2004). CYP1A1 induction is mediated primarily by AHR (Whitlock, 1999). The AHR is a transcription factor with cytosolic expression that is able to sense a wide range of both endogenous and exogenous ligands. The resulting structural composition of the AHR facilitates the translocation of ligand-AHR complexes into nuclei, where they associate with the AHR nuclear translocater (ARNT) and bind to specific deoxyribonucleic acid (DNA) recognition sequences, notably dioxin-response elements or xenobiotic-response elements (XREs), in target genes. This increases the transcription of target genes such as gene encoding for CYP1A1. Subsequently, the affected xenobiotic substance is oxygenated to increase its solubility in water, and undergoes further phase-II conjugation by enzymes such as uridine diphosphate (UDP)-glucuronosyltransferase (UGT) and sulfotransferase (SULT). This process either makes the substrate more polar or eliminates it altogether. However, the oxygenation also initiates the production of active intermediates or procarcinogens that form DNA and protein adducts, leading to tumor formation and toxicity (Ma and Lu, 2007). Besides the AHR-mediated CYP1A induction, the pregnane X receptor (PXR) is also a promiscuous nuclear receptor for both xenobiotic chemicals and endogenous metabolites, and has been reported to coordinate hepatic responses with the constitutive androstane receptor (CAR) and regulate the expression of CYP2 and CYP3 genes to prevent liver injury (Stedman et al., 2005; Johnson et al., 2006).

TH action mediated by TH receptors (TRs) also plays an essential role, as the thyroid gland, the most important endocrine organ, contributes to growth, development and metabolism in vertebrates. TRs may act as either homodimers or heterodimers with the retinoid X receptor (RXR); heterodimer TRs are more functional. The active TH which is T3 (3, 3′,5 triiodo-L-thyronine) can bind TR:RXR heterodimers to activate target gene transcription (Ikeda et al., 1994). If PBDEs mimic TH actions on growth and metabolism, their presence poses a great risk to TH homeostasis and would have significant impacts on growth and development of the affected organisms. A comprehensive picture of the consequences of exposure to PBDEs will have profound implications for cancer research, toxicology, pharmacology and risk assessment.

In a previous study, we used *in vivo* zebrafish embryos/larvae and an *in vitro* ZFL cell line to compare the toxicity of popular brominated flame retardants (BDE-47, -99 and -209 and tetrabromobisphenol A) and bisphenol A (BPA) (Chan and Chan, 2012; Yang and Chan, 2015). Ribonucleic acid (RNA) profiling proved that the target chemicals all disrupt the synthesis, transport and action of THs and the activity of hepatic phase II enzymes. However, the gene differential expression pattern varied between the chemicals. Only BPA consistently down-regulated TR-beta (*trβ*) expression and exhibited an antagonistic effect on T3 induced TRβ luciferase reporter activity. In this study, we performed a time-course analysis of RNA profiling data from ZFL cells exposed to BDE-47 and BDE-99. The extremely strong induction of *CYP1A* by BDE-99 testified to the occurrence of Ahr-mediated CYP1A activation. Nuclear receptor (Pxr, Rxr) luciferase reporter systems were developed to examine other receptor-mediated pathways as well. The data obtained in this study will aid the characterization of molecular disorders caused by PBDEs in fish, and help to delineate better models for assessing the chronic effects of environmental pollutants in aquatic systems.

2. Materials and methods

2.1. References for gene names

The gene names (mRNA and proteins) used in this study are as proposed by Kubota et al. (2015). All mRNAs and proteins in the zebrafish cytochrome P450 family are denoted as *CYP* and CYP, following Nelson et al. (1996). The names of other zebrafish mRNAs and proteins follow the approved guidelines for zebrafish, *e.g.*, *ahr* and Ahr (https://wiki.zfin.org/display/prot/Conventions+For+Naming+Zebrafish+Genes). When not referring to particular species, capitalized abbreviations are used, *e.g.*, *AHR* and AHR.

2.2. Chemicals and solutions

The structures of the BDE congeners and positive-control chemicals in the assays are shown in Fig. 1. BDE-47 (CAS 5436-43-1, 100% purity) and BDE-99 (CAS 60348-60-9, 99.2% purity) were purchased from AccuStandard Inc. (New Haven, CT). Benzo[*a*]pyrene (BaP, CAS B1760), clotrimazole (CLO, CAS C6019) and resorufin ethyl ether (7-ER, CAS 46121) were purchased from Sigma-Aldrich Co. (St. Louis, U.S.A). Stock solutions were prepared in dimethyl sulfoxide (DMSO) and stored at room temperature for use within 1 year. The 7-ER stock was stored at −20 °C. The 9-*cis*-retinoic acid (9C) stock in an ethanol carrier was a gift from Dr. Susanna Lee (School of Life Sciences, The Chinese University of Hong Kong), and was stored at −20 °C. All of the chemicals were of analytical grade, with the highest purity available. Prior to each experiment, the chemicals were prepared in solvent (DMSO or ethanol) then immediately diluted in a serum-free medium to their working concentrations. The final concentration of solvent (DMSO or ethanol) in the test solutions did not exceed 0.1%.

2.3. Cell culture

The ZFL cell line (CRL-2643TM) is an adherent tissue hepatocyte cell line isolated from zebrafish (*Danio rerio*), with an epithelial-like morphology, purchased from the American Type Culture Collection (ATCC). ZFL cells were grown in a standard culture medium comprising 50% L-15 medium, 35% Dulbecco Modified Eagle's Medium and 15% Hams F12 and supplemented with 1.5 g/L sodium bicarbonate, 15 mM Hepes, 5% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin, all purchased from Invitrogen-Gibco (Cheuk et al., 2008; Yang and Chan, 2015). The culture medium was maintained at 28 °C, as recommended by the ATCC.

2.4. Cytotoxicity assay

The cytotoxicity of BaP, CLO and 9C was tested using the AlamarBlue assay as described by Yang and Chan (2015).

2.5. Reporter-gene construction

To investigate the mode of action of BDE-99, the following four luciferase activity based reporter gene systems were constructed.

1. An Ahr dependent xenobiotic response reporter (3XRE-luc).
2. A zebrafish Pxr ligand binding domain (LBD) construct (zfPxr-LBD).
3. An LBD construct for the zebrafish Rxr: subtype β, isoform a (zfRxrba-LBD).
4. An LBD construct for the zebrafish Rxr, subtype β, isoform b (zfRxrbb-LBD).

To construct the 3XRE-luc reporter, the following single-strand oligonucleotide (oligo) containing three repeated core sequences of zfXRE (bold) (Zeruth and Pollenz, 2007) was designed: 5′-CGGGGTACCGCTAAAGCA**TC**GCGTGC**A**A**AG**GTGT GCTAAAGCA**TC**GCGTGC**A**A**AG**GTGTGCTAAAGCA**TC**GCGTGC**A**A**AG**GTGT-CCGCTCGAG-3′. The oligo and its complementary strand were synthesized by Tech Dragon Limited, Hong Kong. Duplex DNA was produced by mixing, heating to 95 °C and cooling to 25 °C. The vector pGL4.17-TATA [Luc2/Neo/minP] was a gift from Prof. K. F. Lau (School of Life Sciences, The Chinese University of Hong Kong). The synthesized 3XRE fragment was cloned into the *KpnI*/*XhoI* sites of pGL4.17-TATA. The