Ecotoxicology and Environmental Safety 142 (2017) 150–156

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

In vitro and *in vivo* estrogenic activity of BPA, BPF and BPS in zebrafish-specific assays

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ARTICLE INFO

Keywords:
Zebrafish
Endocrine disruptors
Bisphenols
Estrogenic activity

ABSTRACT

Bisphenol A (BPA) is a widely used chemical that has been extensively studied as an endocrine-disrupting chemical (EDC). Other bisphenols sharing close structural features with BPA, are increasingly being used as alternatives, increasing the need to assess associated hazards to the endocrine system. In the present study, the estrogenic activity of BPA, bisphenol S (BPS) and bisphenol F (BPF) was assessed by using a combination of zebrafish-specific mechanism-based *in vitro* and *in vivo* assays. The three bisphenols were found to efficiently transactivate all zebrafish estrogen receptor (zfER) subtypes in zebrafish hepatic reporter cell lines (ZELH-zfERs). BPA was selective for zfERα while BPS and BPF were slightly more potent on zfERβ subtypes. We further documented the estrogenic effect *in vivo* by quantifying the expression of brain aromatase using a transgenic *cyp19a1b*-GFP zebrafish embryo assay. All three bisphenols induced GFP in a concentration-dependent manner. BPS only partially induced brain aromatase at the highest tested concentrations (> 30 μM) while BPA and BPF strongly induced GFP, in an ER-dependent manner, at 1–10 μM. Furthermore, we show that BPF strongly induced vitellogenin synthesis in adult male zebrafish. Overall, this study demonstrates the estrogenic activity of BPA, BPF and BPS in different cell- and tissue-contexts and at different stages of development. Differences between *in vitro* and *in vivo* responses are discussed in light of selective ER activation and the fate of the compounds in the models. This study confirms the relevance of combining cellular and whole-organism bioassays in a unique model species for the hazard assessment of candidate EDCs.

1. Introduction

Bisphenol A [BPA, 2,2,-bis(4-hydroxyphenol)propane] is one of the man-made chemicals with the highest volume of production, due to its wide use and great variety of applications (review by Michalowicz, 2014). An extensive amount of literature now demonstrates that BPA is an endocrine disrupting compound (EDC), leading to potential adverse health effects in human (review by Rochester, 2013) and wildlife (review by Oehlmann et al., 2009). Consequently, the use of BPA in food contact materials has been restricted or banned in several countries, and it is becoming evident that other bisphenols, intended to replace BPA for various industrial applications, are increasingly being used and detected in our environment. The question of the endocrinal effects of these substitutes is a current issue in human and environmental health studies.

BPS (2,2-bis [4-hydroxyphenol]sulfone) and BPF (2,2-bis [4-hydroxyphenol]methane) are among the main possible substitutes of BPA. BPS has been found in beverage and food cans (Vinas et al., 2010) and in thermal receipt papers (Becerra and Odermatt, 2012). It has been identified in more than 81% of urinary samples of the American population (Liao et al., 2012a). Recent studies have also reported significant environmental concentrations of bisphenols in aquatic ecosystems. BPF and BPA were reported to be predominant contaminants (Careghini et al., 2015; Liao et al., 2012b; Yamazaki et al., 2015), while increasing concentrations of BPS have been detected in sediment sections that represent the past decade, which is consistent with its

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http://dx.doi.org/10.1016/j.ecoenv.2017.04.009
Received 8 February 2017; Received in revised form 22 March 2017; Accepted 5 April 2017
Available online 11 April 2017
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recent introduction (Liao et al., 2012b).

Due to their structural similarity with BPA, it can be expected that BPS and BPF would share some of the effects of BPA. Up to now, most of the data related to the ED potency of BPS and BPF have been obtained in mammalian *in vitro* models. These data suggest that BPS and BPF are able to interfere with multiple nuclear receptors (Kitamura et al., 2005; Molina-Molina et al., 2013; Delfosse et al., 2012), to induce cell proliferation (Molina-Molina et al., 2013) or to alter testosterone secretion in fetal testis assay (Eladak et al., 2015). *In vivo* studies using mammalian and non-mammalian models are rarer but have shown that these compounds can impact the expression of hormone-regulated genes, producing adverse developmental and reproductive effects (Ji et al., 2013; Kinch et al., 2015; Naderi et al., 2014; Cano-Nicolau et al., 2016).

Among the molecular targets of BPA, its interaction with estrogen receptors (ER) is a well-established mechanism in different biological models. Furthermore, BPA and some congeners have been shown to exhibit differential binding affinities and activities depending on human ER subtypes, *i.e.* ERα and ERβ (Routledge et al., 2000; Delfosse et al., 2012; Molina-Molina et al., 2013). In contrast to humans, teleosts possess three ER subtypes, namely ERα, ERβ1 and ERβ2 (Menuet et al., 2002), which are differently expressed and regulated by estrogens. Because the measured estrogenic activity is known to be influenced by the species of origin, the cellular and the tissular context (Matthews et al., 2000) as well as the developmental stage of organisms, the effect of bisphenols on fish-specific models needs to be assessed.

In this study, we aimed at further investigating the capacity of BPA and its major substitutes to transactivate ERs in a model fish species, the zebrafish, a recognized model organism for investigating endocrine disruption (Segner, 2009). For this purpose, we used a set of *in vitro* reporter gene assays based on stable expression of subtypes of zebrafish ER (*i.e.* zfERα, zfERβ1, and zfERβ2) coupled to estrogen response element (ERE)-driven luciferase in a zebrafish liver cell line (ZFL) that was previously developed (Cosnefroy et al., 2012). Estrogenic activity of bisphenols was further evaluated by using an *in vivo* zebrafish embryo assay (EASZY assay) based on the *cyp19a1b-GFP* transgenic line expressing the Green Fluorescent Protein (GFP) under the control of the ER-regulated cyp19a1b gene in the brain (Brion et al., 2012). Finally, *in vivo* induction of vitellogenin synthesis (VTG) in male fish was examined to further assess the estrogenic activity in a fish liver context.

2. Materials and methods

2.1. Chemicals and cell culture reagents

2,2-Bis(4-hydroxyphenyl)propane (BPA, CAS # 80-05-7), 4,4′-sulfonyldiphenol (BPS, CAS # 80-09-1), bis(4-hydroxyphenyl)methane (BPF, CAS # 620-92-8), 17β-estradiol (E2, CAS #r 50-28-2), 17α-ethynylestradiol (EE2, CAS # 57–63-6) were purchased from Sigma-Aldrich (Quentin Fallavier, France). Leibovitz 15 culture medium (L15), 4-(2-hydroxyethyl) − 1-piperazineethanesulfonic acid (HEPES), F-12 nutrient mixture (Ham's F12) powder, Dulbecco's Modified Eagle Medium High Glucose (DMEM HG) powder, G418, hygromycin B, penicillin and streptomycin were purchased from Fischer Scientific (Illkirch, France). Fetal calf serum (FCS) and epidermal growth factor (EGF) were purchased from Sigma-Aldrich (Quentin Fallavier, France) and sodium bicarbonate and insulin from Dominique Dutscher (Brumath, France). ICI 182–780 (ICI) was purchased from Tocris (Bristol, UK) and flutamide (FLU) was obtained from Sigma-Aldrich (Quentin Fallavier, France).

2.2. Cell culture, chemical exposure and luciferase induction assay

ZELH-zfERs cell lines were previously established by a two-step stable transfection of the zebrafish hepatic cell line ZFL (ATCC CRL-2643) with the luciferase gene under the control of ERE (yielding ZELH cells) and each of the three zebrafish estrogen receptors yielding ZELH-zfERα, ZELH-zfERβ1 and ZELH-zfERβ2 cell lines (Cosnefroy et al., 2012; Sonavane et al., 2016). Cells were routinely cultured at 28 °C in a LDF-SVF culture medium, exactly as previously described (Cosnefroy et al., 2012). For chemical exposure, cells were cultured in phenol red free LDF with dextran-coated charcoal treated serum. The cells were seeded in 96-well plates at 25,000 cells per well for ZELH-zfERα and ZELH-zfERβ1, and at 75,000 cells per well for ZELH-zfERβ2, and were cultured for 24 h before exposure. BPA, BPS and BPF were freshly diluted in dimethyl sulfoxide (DMSO) to get a range of final concentrations from 3 nM to 10 μM in culture medium and a final concentration of solvent of 0.1% v/v. After 72 h of exposure, luciferase activity was assessed in living cells as previously described (Cosnefroy et al., 2012). Results were expressed as a percentage of maximal luciferase induced by E2 10 nM.

2.3. Zebrafish embryos, chemical exposure and in vivo imaging

Newly fertilized *cyp19a1b*-GFP transgenic zebrafish (*Danio rario*, from AB strain) eggs were collected from the breeding stock of adult zebrafish, which were maintained under controlled photoperiod (14/10 h light/dark cycle) in charcoal filtrated water at 28 ± 1 °C and fed with SDS-400 (Special Diet Services, Dietex, Argenteuil, France) twice a day and live brine shrimp (Artemia spp.; Ocean Nutrition). Chemical exposures were performed from 0 to 4 days post-fertilization (dpf) in an incubator at 28 °C under semi-static conditions with a total renewal of the medium each day. Each condition consisted of a minimum of 10 embryos in 100 ml of water contaminated by the diluted chemical in DMSO to get a final solvent concentration of 0.01% v/v. Zebrafish embryos were exposed to a range of concentrations of BPA, BPS or BPF. For each chemical, zebrafish embryos were co-exposed with either ICI (1 μM) or FLU (1 μM). Ethinylestradiol (EE2) 0.05 nM was used as a positive control. At the end of exposure, transgenic zebrafish larvae were collected for fluorescence measurement by image analysis. Each larva was observed and photographed in dorsal view under a fluorescence microscope combined with an AxioCam Mrm camera (Zeiss GmbH, Göttingen, Germany). All photographs were taken with the same parameters and analyzed using Axiovision Imaging Software, as previously described (Brion et al., 2012). Fluorescence quantification was based on the measurement of the integrated density of the region of interest for each picture using ImageJ software as previously described (Brion et al., 2012). Results were expressed as mean fold of induction above control.

2.4. BPF exposure and plasmatic vitellogenin measurement in adult zebrafish

Six-month-old male adult zebrafish (*Danio rerio*, AB strain) were exposed to BPF (0.1, 1 and 10 μM) or E2 (10 nM), using DMSO as the vehicle (concentration in water of 0.006%, v/v). For every condition, eight zebrafish were exposed for 7 days in 4 l of water with a total water renewal every 24 h. At the end of exposure, fish were euthanized. Blood (5 μl) was collected *via* a micropipette tip through a ventral incision between the abdomen and the head and stored at − 80 °C. Vitellogenin concentrations were measured using a competitive zebrafish vitellogenin enzyme linked immune-sorbent assay (zf-Vtg ELISA) as previously described (Brion et al., 2002). Results were expressed as a mean with 95% confidence interval, in ng per ml of plasmatic vitellogenin and analyzed using the Mann-Whitney test. Animal handling and experimentations were in accordance with the EU Directive 2010/63/EU for animal experiments.

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