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Differential activity of BPA, BPAF and BPC on zebrafish estrogen receptors *in vitro* and *in vivo*

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Abstract

The high volume production compound bisphenol A (BPA) is of environmental concern largely because of its estrogenic activity. Consequently, BPA analogues have been synthesized to be considered as replacement molecules for BPA. These analogues need to be thoroughly evaluated for their estrogenic activity. Here, we combined mechanism zebrafish-based assays to examine estrogenic and anti-estrogenic activities of BPA and two of its analogues, bisphenol AF (BPAF) and bisphenol C (BPC) *in vitro* and *in vivo. In vitro* reporter cell lines were used to investigate agonistic and antagonistic effects of the three bisphenols on the three zebrafish estrogen receptors. The transgenic *Tg(5×ERE:GFP)* and *Cyp19a1b-GFP zebra*fish lines were then used to analyze estrogenic and anti-estrogenic responses of the three bisphenols *in vivo*. BPA, BPAF and BPC were agonists with different potencies for the three zebrafish estrogen receptors *in vitro*. The potent zfERα-mediated activity of BPA and BPAF *in vitro* resulted *in vivo* by activation of GFP expression in zebrafish larvae in the heart (zfERα-dependent) at lower concentrations, and in the liver (zfERβ-dependent) at higher concentrations. BPC induced zfERβ-mediated luciferase expression *in vitro,* and the zfERβ agonism led to activation of GFP expression in the liver and the brain *in vivo*. In addition, BPC acted as a full antagonist on zfERα, and completely inhibited estrogen-induced GFP expression in the heart of the zebrafish larvae. To summarize, applying a combination of zebrafish-based *in vitro* and *in vivo* methods to evaluate bisphenol analogues for estrogenic activity will facilitate the prioritization of these chemicals for further analysis in higher vertebrates as well as the risk assessment in humans.

Keywords: zebrafish, bisphenol, estrogen receptor, transgenic fish, reporter cell lines

Introduction

The environmental pollutant bisphenol A (BPA) has been widely studied during the past decade. BPA has since the 1950s been used to make epoxy resin and polycarbonate plastics, and it is one of the highest volume chemicals produced worldwide. BPA is found in products such as reusable food and beverage containers, lining of cans, dental materials, computers, and thermal paper. Through its estrogenic activity, BPA has been shown to cause deleterious effects in laboratory animals, such as abnormal fetal development (Palanza *et al.*, 2008), alterations in sex differentiation (Rubin *et al.*, 2006), reproduction (Honma *et al.*, 2002), brain function and mood (Leranth *et al.*, 2008; Elsworth *et al.*, 2013) as well as promotion of certain types of cancers (Markey *et al.*, 2001; Munoz-de-Toro *et al.*, 2005; Ho *et al.*, 2006). In humans, epidemiological studies have shown that high BPA levels are linked to heart disease, diabetes and liver toxicity ((Lang *et al.*, 2008) and reviewed in (Vandenberg *et al.*, 2012)). Food, indoor air, indoor dust, wastewater and dermal absorption are all important sources of human exposure to BPA (Vandenberg *et al.*, 2007; Zalko *et al.*, 2011). In addition, BPA is commonly found as a contaminant in wastewater, and may as such have deleterious effects on wildlife (Santos *et al.*, 2016; Xu *et al.*, 2016).

Concurrent with the increasing awareness of potential health problems linked to BPA, a search for safer alternatives has begun. Two of these BPA alternatives, bisphenol AF (BPAF) and bisphenol C (BPC), are the focus of this study. BPAF is a fluorinated derivative of BPA, and it is used both as a crosslinking agent and for polymer synthesis in plastics industry. It has an annual estimated production of 10,000–500,000 pounds in the United States (NTP-NIEHS, 2008). BPAF has been detected in wastewater from water treatment plants and was also shown to be

resistant to biodegradation (Sun *et al.*, 2017). BPC is used in the preparation of flame-resistant polycarbonates, and it has also been detected in wastewater (Cesen *et al.*, 2018).

In humans, BPA interferes with estrogen signaling mediated by the two estrogen receptors, ERα and ERβ. The canonical estrogen signaling pathway involves estrogen binding to the ligand binding domain (LBD) of the ERs, translocation of the ER-ligand complexes into the nucleus, followed by recruitment of co-regulator complexes and docking to the estrogen response element (ERE) of target gene promoters (reviewed in (Nilsson *et al.*, 2001)). Similarly to BPA, *in vitro* studies show that several bisphenol analogues are able to bind and activate the human ERs [(Delfosse *et al.*, 2012; Delfosse *et al.*, 2014; Grimaldi *et al.*, 2019) and reviewed in (Chen *et al.*, 2016)]. BPAF has been repeatedly reported to activate ER-dependent transcription at lower concentrations than BPA and other bisphenol derivatives *in vitro* ((Delfosse *et al.*, 2012) and reviewed in (Chen *et al.*, 2016)). While fewer studies have investigated BPC, this chlorinated analogue has been described to have stronger affinity for the ERs than BPA, acting as a full agonist on MCF7 proliferation, and as both an agonist and antagonist in HELN (HeLa ER negzative cells stably transfected by the ERE-Luc plasmid) cells stably-expressing the human ERs (HELN hERs cells) (Delfosse *et al.*, 2012).

Zebrafish (*Danio rerio*) is an *in vivo* model to study environmental pollutants. There are three ERs (zfERα, zfERβ2 and zfERβ1) in zebrafish, with an approximate 50% amino-acid sequence identity to the human ER orthologues (Menuet *et al.*, 2002). We have previously generated cell lines stably-transfected with the zfER subtypes to study the potential estrogenic activity of environmental pollutants in zebrafish (Pinto *et al.*, 2014). We demonstrated with these cell models that mammalian estrogenic ligands also modulate the activity of the zfERs, although the selectivity and potency of the ligand often varied for the human and zebrafish orthologues. In

zebrafish, the three ERs are all expressed from embryonic stages to adulthood, but with different temporal and organ distributions (Bardet *et al.*, 2002; Chandrasekar *et al.*, 2010; Mouriec et al., 2009). zfERα is detected in the heart valves of the developing zebrafish larvae, while the zfERβ1 subtype is robustly expressed in the liver (Gorelick *et al.*, 2014). In the developing brain, zebrafish embryos express detectable levels of zfERβ2 and zfERβ1, but not zfERα, in different brain regions. The expression of cyp19a1b gene was temporally correlated with that of zfERβ2 and zfERβ1 (Mouriec et al., 2009) suggesting that these receptors drive the cyp19a1b expression.

During the past few years, several transgenic zebrafish models have been developed to investigate the cell- and/or tissue-specific effects of chemicals on estrogen signaling pathways in developing fish. For instance, in transgenic zebrafish model containing five EREs (*Tg(5×ERE:GFP))* (Gorelick and Halpern, 2011), the GFP expression is clearly detected in tissues expressing the estrogen receptors. Thus, compounds with *in vitro* selectivity for zfERα and zfERβs induce expression of the fluorescent protein in the heart and liver, respectively (Gorelick *et al.*, 2016). For example, PPT specifically activates the zfERβs receptors *in vitro* and induces GFP expression only in the liver of zebrafish larvae, whereas the pan-agonist 17α-ethinylestradiol induces GFP in both heart and liver (Gorelick and Halpern, 2011; Pinto *et al.*, 2014; Gorelick *et al.*, 2016). Another reporter fish model, the transgenic *cyp19a1b-GFP* zebrafish, expresses GFP under the control of the ER-regulated cyp19a1b (brain aromatase) gene in radial glial cells in the brain (Tong et al., 2009). This fish model has been thoroughly evaluated for assessing the estrogenic activity of a panel of environmental contaminants - including natural and synthetics hormones, pharmaceuticals, pesticides, industrial chemicals- in embryos and allowed to demonstrate that numerous contaminants were able to disrupt the brain-

specific expression of the cyp19a1b gene in an ER-dependent manner (Brion *et al.*, 2012; Cano-Nicolau, et al., 2016; Le Fol et al., 2017).

In this article, we combined *in vitro* and *in vivo* zebrafish mechanism-based reporter gene assays as screening tools to assess the estrogenic properties of BPA, BPAF and BPC. HELN cells expressing the zfERs (HELN zfERs cells) were first used to evaluate the capacity of the selected bisphenols to bind to the three zfERs subtypes and to transactivate zfERs to induce luciferase activity. To further evaluate their estrogenic properties at the organism level, (*Tg(5×ERE:GFP)) and tg(cyp19a1b-GFP)* zebrafish embryos were exposed to BPA, BPAF and BPC and the tissue-specific estrogenic responses in the brain, heart and liver of zebrafish embryos were observed and/or quantified and interpreted in light of their in vitro estrogenic activities towards the zfER subtypes.

Materials and Methods

Materials

Tissue culture plates were obtained from Greiner Bio-one (Monroe, NC, USA), and media was purchased from Invitrogen (Grand Island, NY, USA). Luciferin (sodium salt) was purchased from Promega (Charbonnières, France). The chemicals 17β-estradiol (E2), 17α-ethinylestradiol (EE2), bisphenol A (CAS no. 80-05-7), bisphenol AF (BPAF; CAS no. 1478-61-1), and bisphenol C (BPC; CAS no. 14868-03-2) were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). ICI 182,780 (7α,17β-[9[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol, fulvestrant) was obtained from Zeneca (Macclesfield, UK), and 3,17-Dihydroxy-19-nor-17α-pregna-1,3,5(10)-triene-21,16α-lactone (16α-LE2) was a kind gift of

Peter Muhn from Research Laboratories of Schering AG (Berlin, Germany). Compounds were dissolved in dimethyl sulfoxide (DMSO) as 10^{-2} M stock solutions.

Cell lines

The establishment and culturing of the HELN zfERα (Esr1), zfERβ1 (Esr2b) and zfERβ2 (Esr2a) reporter cell lines have been previously described (Pinto et al. 2014). Briefly, the HELN-zfERα, β1 and β2 cell lines were obtained by transfection of HELN cells (HeLa cells stably transfected with the ERE-βGlobin-Luc-SVNeo plasmid) (Delfosse *et al.*, 2012) by the corresponding pSG5-puro plasmids (pSG5-zfERα-puro, pSG5-zfERβ1-puro and pSG5-zfERβ2-puro, respectively).

The HELN zfER cells were maintained in phenol red-free Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) and supplemented with 5% dextran-coated charcoal-treated fetal bovine serum (FBS-DCC), 1% antibiotic (penicillin/streptomycin), geneticin (1 mg/mL) and puromycin (0.25 µg/mL) in a 5% CO_2 humidified atmosphere at 37°C.

zfERs binding assays

The assay for measuring zfER binding affinity has been previously described (Pinto *et al.*, 2014). Briefly, the HELN-zfERs cell lines were treated with 3 nM [^{3}H]-E2 in the absence or in the presence of chemicals for 4h at 28°C. At the end of the incubation period, unbound material was aspirated, cells washed with PBS, and [^{3}H]-bound radioactivity counted. Protein concentrations were used to normalize bound radioactivity values expressed in desintegrations per minute (dpm). Experiments were performed in quadruplicate in at least two independent experiments.

157

158 *Transactivation assays*

159 Chemicals successive dilutions were performed in cell culture medium, using the same protocol
160 for treatments as described previously (Pinto *et al.*, 2014). The compounds were added 8 h after
161 seeding cells into 96-well plates, and cells were incubated with compounds for 16h at 28°C.
162 Luciferase assays were performed as previously published (Pinto *et al.*, 2014), and experiments
163 were performed in quadruplicates in three independent experiments. Data were expressed as
164 percentage of the maximal activity induced by E2 10 nM. EC50 values were measured using
165 GraphPad Prism (GraphPad Software Inc). Datas were analyzed for significant differences using
166 one-way ANOVA followed by Dunnet's post-comparison test (vs.control) (***$p<0.001$,
167 **$p<0.01$, *$p<0.05$). Differences were considered statistically significant at $P<0.05$.

168

169 *Zebrafish maintenance and transgenic zebrafish Tg(5xERE:GFP) treatment*

170 Transgenic fish *Tg(5×ERE:GFP)* (Gorelick and Halpern, 2011) were used according to the
171 maintenance and experimental protocols approved by the Institutional Animal Care and Use
172 Committee at the University of Houston. Adult zebrafish were maintained and fed as described
173 previously (Hao *et al.*, 2013).

174 Embryos from several pairs of adult fish were collected after spawning and cultured in a Petri
175 dish in E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4) at
176 28.5˚C with 14:10 LD. For fluorescent imaging experiments, *Tg(5xERE:GFP)* zebrafish
177 embryos were incubated in E3 medium with 0.003% 1-phenyl-2-thiourea (PTU) (Sigma-Aldrich,

St. Louis, MO) starting at 24h post fertilization (hpf) to inhibit pigment formation. Samples of 10 zebrafish larvae at 3 days post fertilization (dpf) were treated for 24 h with EE2, 16α-LE2 and bisphenols in 24-well plate, and transferred to 96-well plates at a density of one larva per well for imaging. EE2, 16α-LE2, ICI 182,780 and bisphenols in 100% DMSO were diluted in embryo media, and vehicle concentration did not exceed 0.2% DMSO in treatments. Vehicle only was used as a negative control. The larvae were imaged under 10X magnification lenses using a fluorescent microscope (Olympus IX51) equipped with an Olympus XM10 camera (Olympus, Center Valley, PA).

Treatment of cyp19a1b-GFP transgenic zebrafish embryos

Newly fertilized transgenic *cyp19a1b-GFP* zebrafish eggs were collected and treated as previously described (Brion *et al.*, 2012). Briefly, 20 embryos (< 4 h post-fertilization) were exposed from 0 to 4 dpf to DMSO (0.01% v:v), 0.05 nM EE2 (used as positive control) or a range of concentrations of BPA, BPAF or BPC. The exposure medium was renewed totally every 24 h. At the end of the exposure period, the zebrafish embryos were collected for *in vivo* fluorescence imaging of the brain. Each 4 dpf-old zebrafish was photographed in dorsal view under a fluorescence microscope connected to an AxioCam Mrm camera (Zeiss GmbH, Gottingen, Germany). All photographs were taken with the same parameters. The fluorescence of the regions of interest was then quantified by image analysis using an ImageJ macro as previously described (Brion *et al.*, 2012).

RNA extraction and quantitative real-time PCR

Zebrafish larvae were treated with bisphenols and controls from 4 dpf to 6 dpf. Treatments were repeated once at 5 dpf. Total RNA from 40 pooled embryos were collected at 6 dpf using Trizol (Invitrogen Corporation, Carlsbad, CA) and RNeasy spin columns (Qiagen, Chatsworth, CA) according to manufacturer's protocols. DNase I (Qiagen, Chatsworth, CA) was used to remove any DNA in the column. RNA concentration was measured using NanoDrop 1000 spectrophotometer (Agilent Technologies, Palo Alto, CA) and 100 ng of total RNA was used for cDNA synthesis using Superscript III reverse transcriptase (Invitrogen Corporation, Carlsbad, CA) with random hexamer primers.

Expression of Cyp19a1b and Vtg1 were measured by qPCR using an ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA) with iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. Primers were synthesized by Integrated DNA Technologies, Inc (Coralville, IA). Primer sequences were for vtg1 Forward (5'-3') ACTACCAACTGGCTGCTTAC and Reverse (5'-3') ACCATCGGCACAGATCTTC; and for cyp19a1b Forward (5'-3') AAAGAGTTACTAATAAAGATCCACCGGTAT and Reverse (5'-3') TCCACAAGCTTTCCCATTTCA (Hao *et al.*, 2013). Expression levels were normalized to zebrafish β-actin using the ΔΔCt method. Student's t-test was performed for statistical analysis using GraphPad Prism version 7 (GraphPad Software, La Jolla, CA).

Results

Transcriptional activity of bisphenols mediated by zfERs *in vitro*

The transcriptional activity of BPA, BPAF and BPC have been reported for human estrogen receptors in HELN cells (Delfosse et al, 2012). To investigate the activity of BPA and its

analogues on zebrafish ERs, we treated the HELN cell lines expressing the zfERα, zfERβ1 and zfERβ2 receptors and an ERE-driven luciferase reporter with increasing concentrations of the bisphenols. BPA and BPAF activated the three zfER reporter cells, reaching approximately 53-89% of E2-induced luciferase expression (Figure 1A, B). These bisphenols displayed higher selectivity for zfERα, than for the zfERβ subtypes. BPAF was more potent than BPA in inducing luciferase expression, and displayed approximately 3.1-5.6-fold greater potency on the three zfER cells than BPA, with EC50s of 84, 758 and 897 nM for zfERα, zfERβ1 and zfERβ2, respectively (Table 1). BPC activated both zfERα and zfERβ1 at low concentrations, but the luciferase activity reached only 31 and 36% of the maximal luciferase activity, respectively. BPC induced transcription to 59% of maximal activation on zfERβ2 (Figure 1C). The measured EC50s and maximal activation for the BPA, BPAF, BPC and controls are shown in Table 1.

BPA, BPAF and BPC were identified as partial agonists in HELN hERs cells (Delfosse *et al.*, 2012). The partial bisphenols induced-luciferase activity in the human ER reporter cells and the marginal effect of BPC on zfERα and zfERβ1 (figure 1C) suggest that BPC can act as an antagonist for these zfERs. We therefore evaluated the ability of BPC to antagonize the activity of E2. We found that BPC clearly reduced E2-induced transactivation in the zfERα and zfERβ cell lines with IC50s of 46, 160 and 136 nM on zfERα, zfERβ1 and zfERβ2, respectively (Figure 1D; Table 1) thereby revealing a stronger antagonistic activity of BPC towards zfERα and zfERβ1.

The selectivity of BPA, BPAF and BPC for zfERα was confirmed in competitive binding assays with radiolabeled E2 (Table 2). As previously demonstrated for the selective zfERα compound 16α-LE2 (Pinto *et al.*, 2014), bisphenols displayed stronger binding affinity for zfERα

compared to the zfERβ subtypes. Additionally, BPAF and BPC displaced radiolabeled E2 at lower concentrations than BPA, confirming the stronger affinity of BPAF and BPC for the zebrafish ERs relative to BPA (Table 2).

Activation of estrogen signaling by bisphenols *in tg ERE-GFP embryos*

To assess the estrogenic effects of BPA, BPAF and BPC *in vivo* in zebrafish embryos, the transgenic fish line *Tg(5xERE:GFP)* containing 5xERE upstream of a *c-fos* promoter and a GFP reporter was used (Gorelick and Halpern, 2011). In order to qualitatively investigate the tissue-specific activity of the bisphenols, we treated 3 dpf zebrafish larvae for 24 h with BPA, BPAF and BPC (Figure 2). The ligand 16α-LE2, whose zfERα selectivity *in vitro* is associated with *in vivo* GFP expression exclusively in the heart valves at lower doses (10-100 nM) (Figure 2C-D), was used to compare the tissue-specific effects resulting from treatment with the bisphenols. The zfERα-selective agonist activity of BPA and BPAF was confirmed in the zebrafish embryos. At lower concentrations, BPA and BPAF only activated GFP expression in the heart valves, while higher doses of the ligands were required for induction of liver expression (Figure 2G-J and 2K-N). Treatment with BPC induced GFP expression in the liver at 1 µM, and no heart GFP expression was observed, likely due to its only marginal activity on zfERα (Figure 2O-R).

The stronger potency of BPAF and BPC on the zfERs compared to BPA observed *in our experiments vitro* was confirmed *in vivo*. Treatment with 100 nM BPAF induced GFP expression in the heart, whereas a dose of 1 µM BPA was required to achieve a similar effect in the zebrafish embryos. At the hepatic level, GFP expression was induced after exposure to 1 µM BPAF or 1 µM BPC, while BPA was only able to activate hepatic GFP expression at 10 µM

(Figure 2G-R). Therefore, the responses observed in vivo seems to reflect both the estrogenic activity reported in vitro as well as the selectivity of the compounds towards the zfERs. Thus, the stronger potencies of BPAF and BPC compared to BPA seen *in vitro* was also observed at the *in vivo* level.

To investigate the antagonist activity of BPC *in vivo*, we co-treated zebrafish embryos with the estrogenic ligand EE2, which is more stable than E2 *in vivo*, or 16α-LE2 in combination with BPC (Figure 3 and Figure 4). Treatment with 100 pM EE2 induced GFP expression both in the heart and liver of the embryos, in accordance with its pan-agonist ER activity (Figure 3C) (Pinto *et al.*, 2014; Gorelick *et al.*, 2016). The pure antagonist ICI 182,780 completely blocked both heart and liver GFP expression (Figure 3E). Treatment with 1 μM BPC induced GFP expression in the liver of the embryos in accordance with its zfERβ2 activity. Co-treatment of EE2 with 1 μM BPC completely blocked EE2-induced GFP heart expression whereas it only slightly reduced EE2-induced GFP liver expression (Figure 3G). Likewise, BPC was capable of completely inhibiting the GFP expression induced by 10 nM 16α-LE2 in the heart (Figure 4). These results are in accordance with the *in vitro* antagonist/agonist activity of BPC, indicating that BPC is an antagonist on zfERα and partial agonist on zfERβs *in vivo*.

Activation of the brain aromatase promoter in the transgenic cyp19a1b-GFP zebrafish embryos by the three bisphenols

To assess the effect of bisphenols on the expression of the estrogen-sensitive brain aromatase gene, the fluorescence signal in the developing brain of transgenic cyp19a1b-GFP zebrafish embryos was quantified after a 4-day treatment with BPA, BPAF and BPC. All bisphenols

induced GFP expression in the brain of the zebrafish embryos (Figure 5A-C). BPA at 10 µM promoted a 28-fold induction in the GFP signal, whereas no or very weak activity was observed at lower concentrations. Exposure to BPAF or BPC at 1 µM was sufficient to promote a 18-fold induction in the GFP expression, approximately, and a 2.9-fold and 5.5-fold GFP activation was observed at 0.1 µM for BPAF and BPC, respectively (Figure 5A).

To assess the potential for the bisphenols to inhibit estrogen-mediated activation of the brain aromatase promoter, the cyp19a1b-GFP zebrafish embryos were treated with 0.05 nM EE2 in the presence of the bisphenols. Under these experimental conditions, BPA, BPAF and BPC were unable to inhibit the EE2-mediated induction of GFP in the zebrafish embryos. These results indicate that BPA, BPAF and BPC act as agonists to induce brain aromatase expression within the radial glial cell context but that the bisphenols lack of ER antagonist activity on the brain aromatase of zebrafish embryos (Figure 5B-C).

Endogenous estrogen target gene activation by bisphenols *in vivo*

To analyze the effect of BPA, BPAF and BPC exposure on the expression of endogenous estrogen target genes, we performed quantitative PCR. We investigated the expression of the estrogen-sensitive markers vtg1 gene, highly expressed in the liver, and the brain cyp19a1b. In concordance with the *in vivo* GFP expression data, BPAF and BPC activated vtg1 and cyp19a1b at lower concentrations than BPA (clearly detected at 1 µM vs. 10 µM, respectively) (Figures 6A and 6B). We conclude that the *in vivo* endogenous activation of estrogenic target genes by BPA analogs is in accordance with both the brain and liver *in vivo* GFP activation.

Discussion

Evidence in the literature showing that exposure to BPA causes harmful health effects in humans and wildlife has prompted a search for safer bisphenol alternatives. We assessed the effects of BPA, BPAF and BPC using zfER reporter cell lines, and found that BPAF and BPC in comparison to BPA have a stronger binding affinity for the zebrafish ERs, and a lower EC50 to activate them. *In vitro*, BPAF and BPA activated luciferase expression mediated by the three zebrafish estrogen receptors, with stronger affinity for zfERα. This zfERα selectivity was confirmed by the preferential activation of the GFP reporter in the heart valves *in vivo* in zebrafish larvae. BPC only minimally stimulated the activity of zfERα *in vitro*, and partially activated zfERβ-mediated transcription to levels ranging from 40-60% of the activity of E2. In accordance with its agonist profile *in vitro*, exposure to BPC induced GFP expression in the brain and in the liver of the zebrafish larvae. Additionally, the stronger affinity of BPAF and BPC for the zebrafish receptors compared to BPA *in vitro* was confirmed *in vivo*, as lower concentrations of these bisphenol analogues were necessary to activate liver and brain GFP expression, as well as promote the induction of vtg1 and cyp19a1b gene expression in zebrafish embryos.

The estrogenic activities of bisphenols have previously been analyzed for human ERs in reporter cell lines (Kitamura *et al.*, 2005; Matsushima *et al.*, 2010; Delfosse *et al.*, 2012; Chen *et al.*, 2016; Grimaldi *et al.*, 2019). The pronounced agonist activity of BPAF has been previously reported for human ERs, with several studies demonstrating that BPAF is more estrogen-active than BPA. In HELN cells expressing the human ERs, both BPAF and BPC are more potent ligands than BPA (Delfosse *et al.*, 2012). Bisphenols show a slightly better affinity for human ERβ. This opposite ER selectivity profile between human and zebrafish ERs also observed for

several other xenoestrogens, could be due to differences in the constitution and interaction with amino acids in the LBD of human and zebrafish receptors (Pinto *et al.*, 2014).

The *in vivo* estrogenic activity of bisphenols have been studied in fish and rodents. In adult male medaka, BPAF and BPC are more potent than BPA in inducing hepatic upregulation of the expression of vtg and other estrogenic biomarkers (Yamaguchi *et al.*, 2015). Moreover, the bisphenols induce uterine weight gain in ovariectomized rats, and the uterotrophic effect is more pronounced for BPC and BPAF compared to BPA (Conley *et al.*, 2016). These *in vivo* results are in accordance with our results from *in vitro* and transgenic zebrafish models, and provide substantial data to support that BPC and BPAF have a stronger ability than BPA to disrupt estrogen signaling across different vertebrate species.

The present study is, to our knowledge, the first to demonstrate that the chlorinated bisphenol analogue, BPC, has pronounced antagonistic zfERα activity in zebrafish both *in vitro* and *in vivo*. Exposure to BPC resulted in clear inhibition of the EE2- and 16α-LE2-induced GFP expression in the heart of the zebrafish larvae, in accordance with its zfERα-mediated antagonist activity in the HELN reporter cells. The outcomes of activation or inactivation of estrogen signaling by bisphenols on the development and physiology of the zebrafish heart are unknown. However, it has been reported that estrogenic ligands can alter the basal heart rate in zebrafish embryos. Exposure to estradiol and ICI 182,780 resulted in increased heart rate in zebrafish, an effect that was attributed to modulation of the G protein-coupled estrogen receptor (GPER) pathway (Romano *et al.*, 2016), while treatment with the anti-estrogen tamoxifen caused a decrease in the heart rate of zebrafish embryos (Xia *et al.*, 2016). BPA treatment has been shown to impair the cardiac contractile performance of rodent hearts, an effect associated with altered intracellular calcium handling (Posnack *et al.*, 2015).

Although ER antagonist activity was observed for BPC in the ERE-driven activation of fluorescence in the zebrafish heart, BPC, as well as BPA and BPAF, were unable to inhibit the EE2-induced brain aromatase promoter. zfERβ2 has been reported to be the main ER subtype detected in the brain during zebrafish early developmental stages, while zfERα and zfERβ1 mRNA are in contrast much lower (Mouriec *et al.*, 2009). Studies have also shown that morpholino knockdown of zfERβ2, but not zfERα or zfERβ1, completely blocks estrogen-mediated upregulation of the aromatase mRNA (Griffin *et al.*, 2013). This data suggests that zfERβ2 is the main subtype controlling the activation of brain aromatase during zebrafish early development. The results with BPC support this hypothesis, as BPC would be expected to inhibit the estrogen-mediated activation of aromatase in the cyp19a1b-GFP zebrafish embryos if aromatase expression was indeed controlled by zfERα. Inferring from this data, BPC will act as both an agonist and antagonist *in vivo* in a tissue-specific manner.

In addition to perturbing the ER pathway, BPAF and BPC have been reported to interact with other endocrine targets (Delfosse *et al.*, 2014; Grimaldi et al., 2019; Lei et al., 2017; Wang *et al.*, 2014). The increasing and widespread use of these bisphenol analogues and their ability to interact with endocrine molecular targets at lower concentrations than BPA pose a serious concern associated with potential health risks for humans and wildlife. Implementation of green chemistry practices for identification of safer alternatives will improve characterization of chemical hazard and should be useful to guide manufacturers on more sustainable bisphenol alternatives.

In conclusion, we show that a combined strategy using cell-based assays and transgenic zebrafish models can be used to thoroughly assess estrogenic properties of bisphenol derivatives. This

approach allows for the quick screening of both estrogenic and anti-estrogenic activities of environmental pollutants, and are sensitive tools to inform dose-response relationships and tissue-specific effects. Characterization of species differences in response to estrogenic compounds can be addressed in the HELN cells expressing human or zebrafish ERs. The transgenic zebrafish models could also be used to address the influence of metabolism and pharmacokinetic parameters on chemical activity. Moreover, these tools are amenable to high-throughput screening, and can be incorporated into screening platforms currently applied to inform perturbations in ER signaling, such as the ones of the U.S. Environmental Protection Agency (EPA)'s ToxCast/Tox21 program.

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Figure legends

Fig. 1. Agonistic and antagonistic activity of bisphenols in HELN zfER reporter cell lines. (A-C) Dose-response curves for BPA, BPAF, and BPC in HELN zfER reporter cell lines. Cells were incubated for16h with various concentrations of BPA, BPAF, and BPC. (D) Dose responsive curves of BPC in HELN zfER reporter cell lines. Cells were incubated for 16h with various concentrations of BPC in presence of in presence of 1 nM E2. Data were expressed as percentage of the maximal activity induced by E2 10 nM. EC50 values were measured using GraphPad Prism (GraphPad Software Inc). Datas were analyzed for significant differences using one-way ANOVA followed by Dunnet's post-comparison test (vs.control) (***$p<0.001$, **$p<0.01$, *$p<0.05$).

Fig. 2. *In vivo* estrogenic activities of bisphenols on ERE reporter fish. *Tg (ERE:GFP)* larvae were treated with 16α-LE2 or bisphenols at 3 dpf for 24 h. Images were taken at 4 dpf. Lateral view showing GFP expression in the heart valves (red arrows) and liver (white arrow). A. Bright-field image; B. GFP fluorescence image of vehicle-treated zebrafish larvae; C-F. GFP images of zebrafish larvae exposed to increasing concentrations of 16α-LE2; G-J. GFP images of

zebrafish treated with BPA; K-N. Images of zebrafish exposed to BPAF; O-R. GFP images of zebrafish exposed to BPC. *Scale bars*, 200 μm.

Fig. 3. Anti-estrogenic effects of BPC on ERE reporter fish co-treated with EE2. A. Bright-field image; B. GFP fluorescence image of vehicle-treated zebrafish larvae; C. GFP images of zebrafish larvae exposed to 100 pm EE2; D. Zebrafish exposed to 1 μM ICI 182,780; E. GFP images of zebrafish co-treated with EE2 and ICI; F. Images of zebrafish exposed to 1 μM BPC; G. GFP images of zebrafish co-treated with EE2 and BPC. *Scale bars*, 100 μm.

Fig. 4. Anti-estrogenic effects of BPC on ERE reporter fish co-treated with 16α-LE2**.** A. Bright-field image; B. GFP fluorescence image of vehicle-treated zebrafish larvae; C. GFP images of zebrafish larvae exposed to 10 nM 16α-LE2; D. GFP images of zebrafish exposed to 3 μM BPC; E. GFP images of zebrafish larvae co-treated with 16α-LE2 and BPC. *Scale bars*, 200 μm.

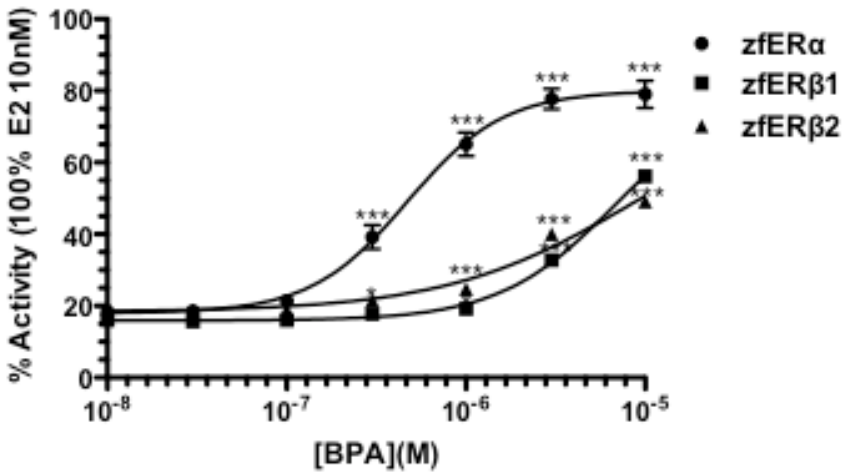
Fig. 5. *In vivo* estrogenic activity of bisphenols in the *cyp19a1b-GFP* zebrafish. A. Concentration-dependent induction of GFP by BPA, BPAF and BPC in *cyp19a1b-GFP* transgenic larvae after exposure to the bisphenols from 0 to 4 dpf. Values are expressed as mean +/- SEM (n = 4-13). B. Effect of BPA, BPAF and BPC on EE2-mediated GFP induction in *cyp19a1b*-GFP transgenic larva after exposure from 0 to 4 dpf. Values are expressed as mean +/- SEM (n = 8-14). C. Fluorescence images of *cyp19a1b-GFP* transgenic larvae treated with 0.05

577 nM EE2, 10 µM BPA, 1 µM BPAF and 1 µM BPC alone and co-treatments of EE2 with the
578 bisphenols. Images were taken in the dorsal view, anterior side to the top.

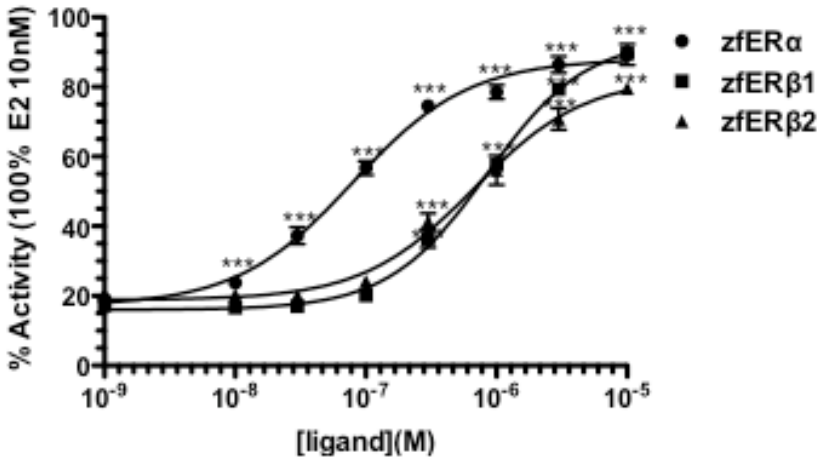
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580 Fig. 6. Endogenous estrogen target gene activation by bisphenols *in vivo.* Concentration-
581 dependent induction of *vtg1* (A) and *cyp19a1b* (B) mRNA levels after treatment of zebrafish
582 embryos with BPA, BPAF and BPC from 4 to 6 dpf. Transcript levels were normalized to
583 zebrafish β-actin using the ΔΔCt method.

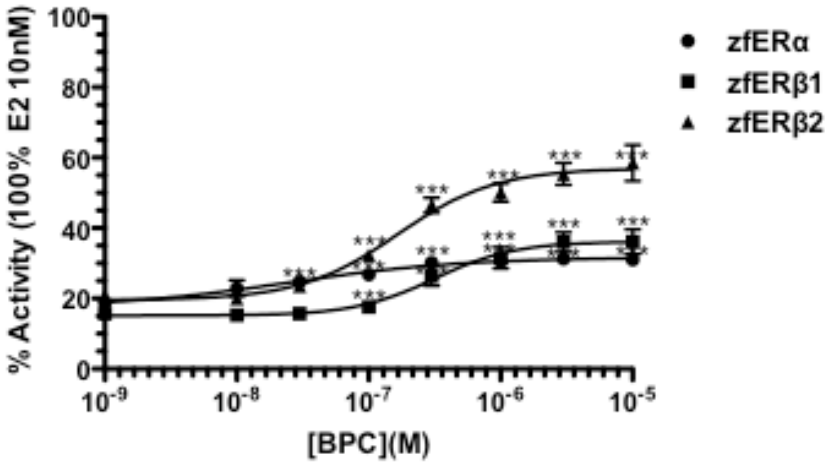
1A



1B



1C



1D

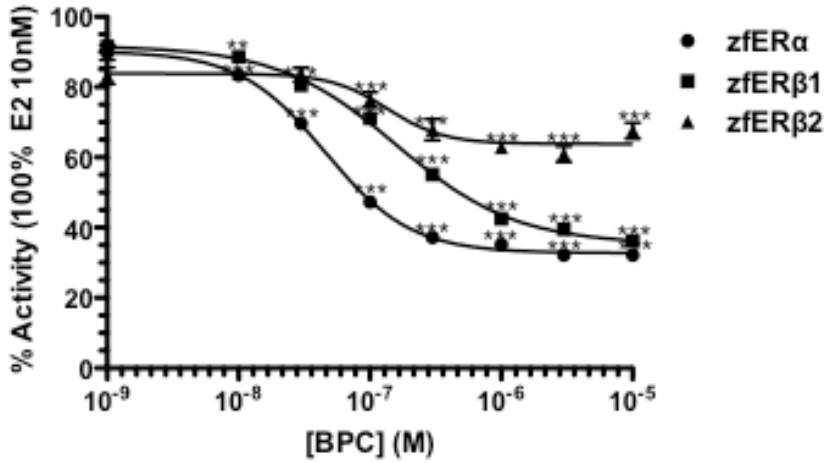


Figure 1

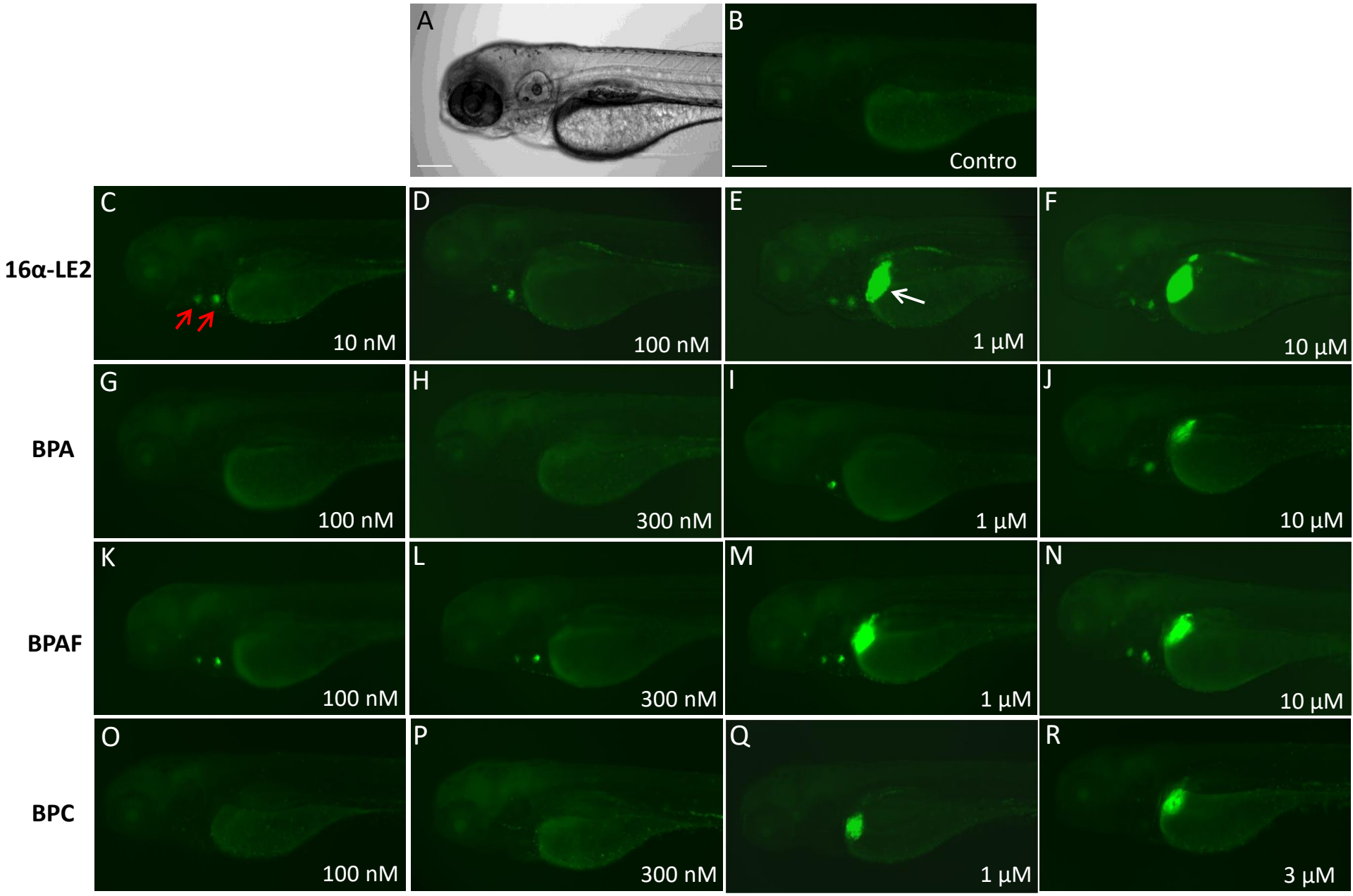

A
B
Contro
16α-LE2
C
10 nM
D
100 nM
E
1 μM
F
10 μM
BPA
G
100 nM
H
300 nM
I
1 μM
J
10 μM
BPAF
K
100 nM
L
300 nM
M
1 μM
N
10 μM
BPC
O
100 nM
P
300 nM
Q
1 μM
R
3 μM

Figure 2

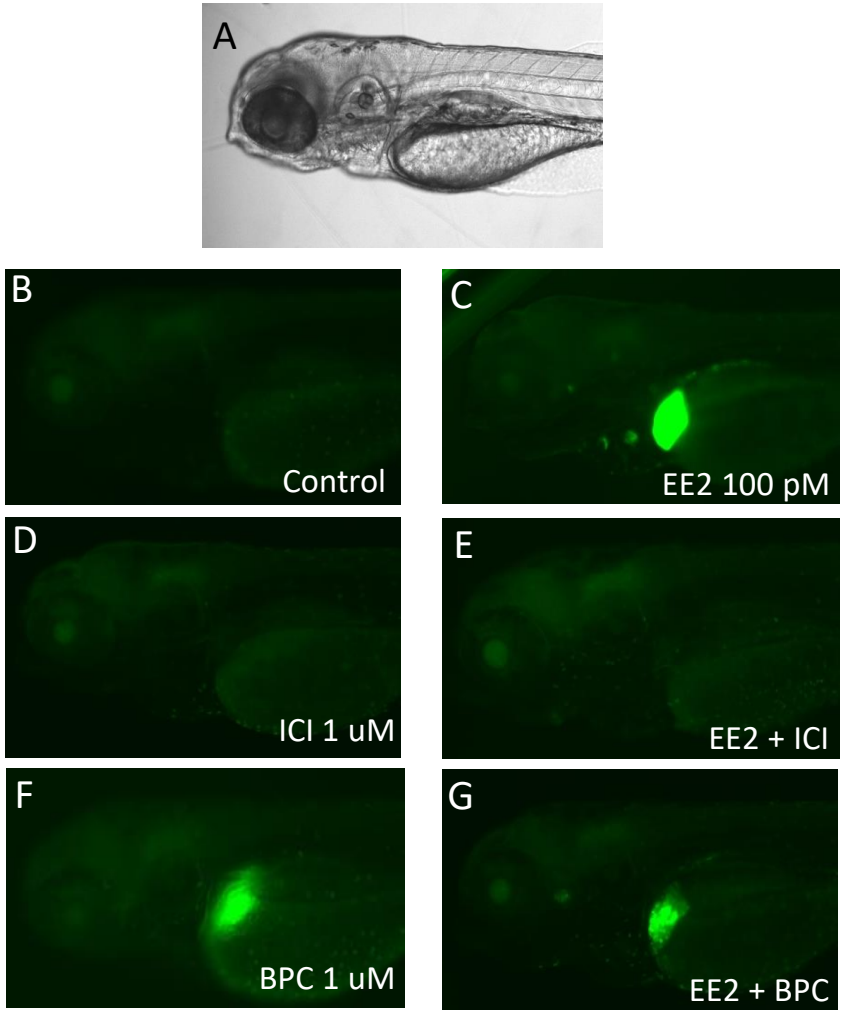


Figure 3

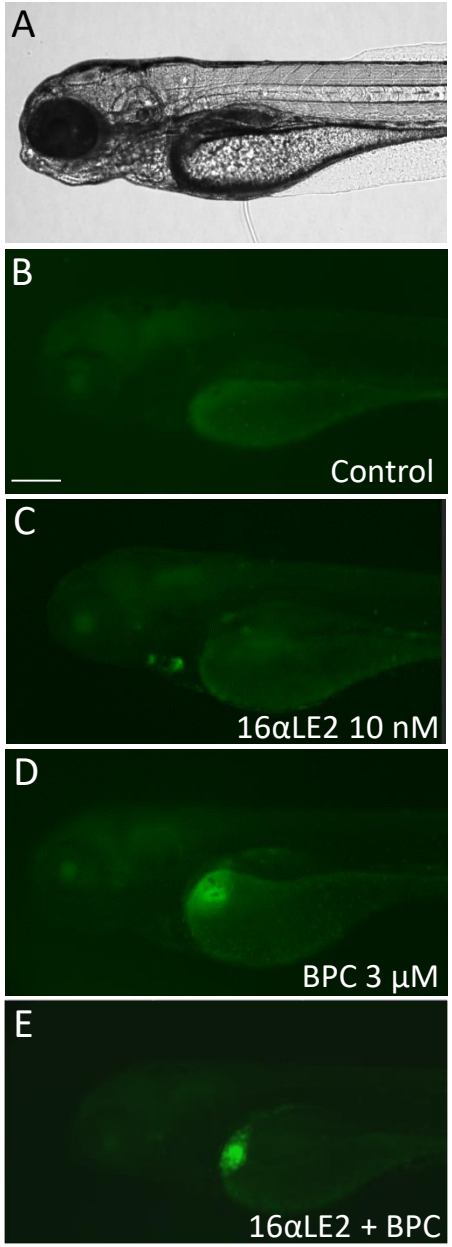
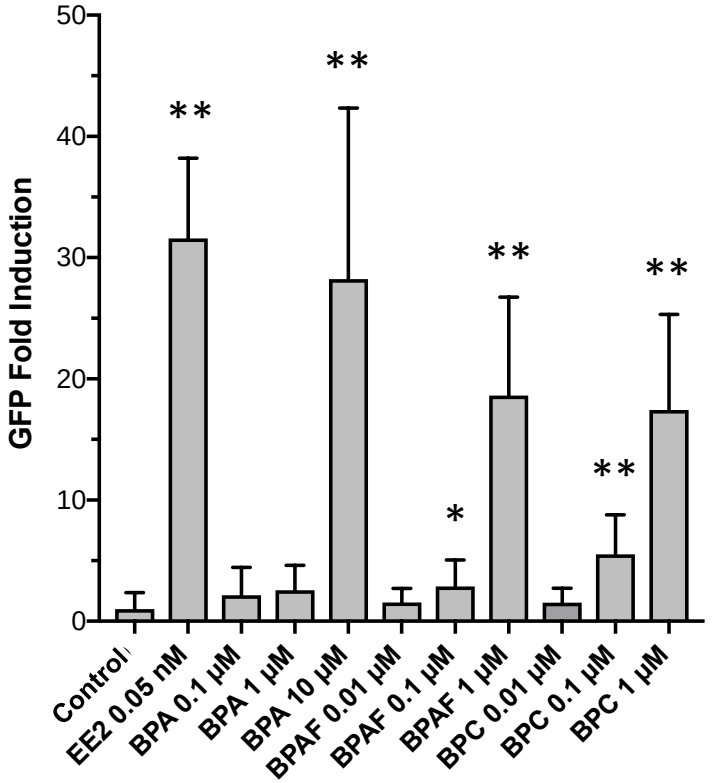
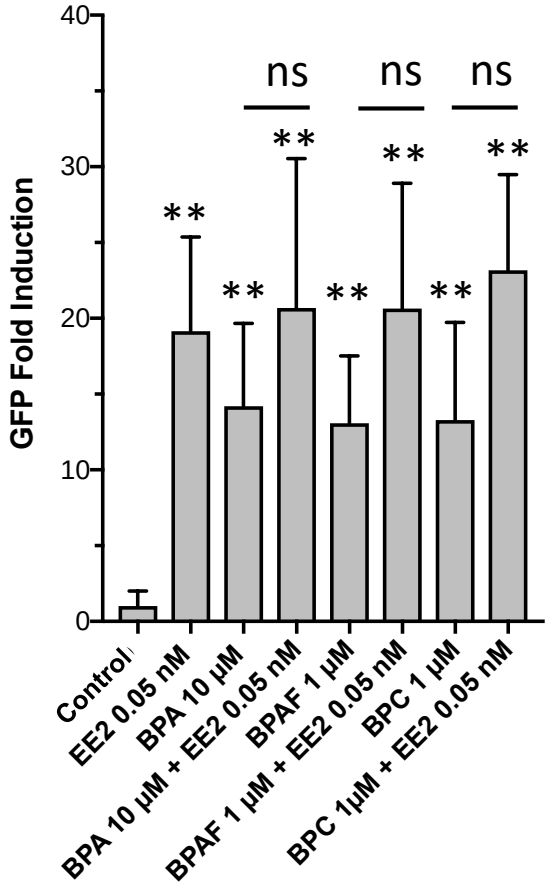


Figure 4

A


GFP Fold Induction
50
40
30
20
10
0
**
**
**
*
**
**
Control
EE2 0.05 nM
BPA 0.1 µM
BPA 1 µM
BPA 10 µM
BPAF 0.01 µM
BPAF 0.1 µM
BPAF 1 µM
BPC 0.01 µM
BPC 0.1 µM
BPC 1 µM

B


GFP Fold Induction
40
30
20
10
0
ns
ns
ns
**
**
**
**
**
**
Control
EE2 0.05 nM
BPA 10 µM
BPA 10 µM + EE2 0.05 nM
BPAF 1 µM
BPAF 1 µM + EE2 0.05 nM
BPC 1 µM
BPC 1µM + EE2 0.05 nM

C

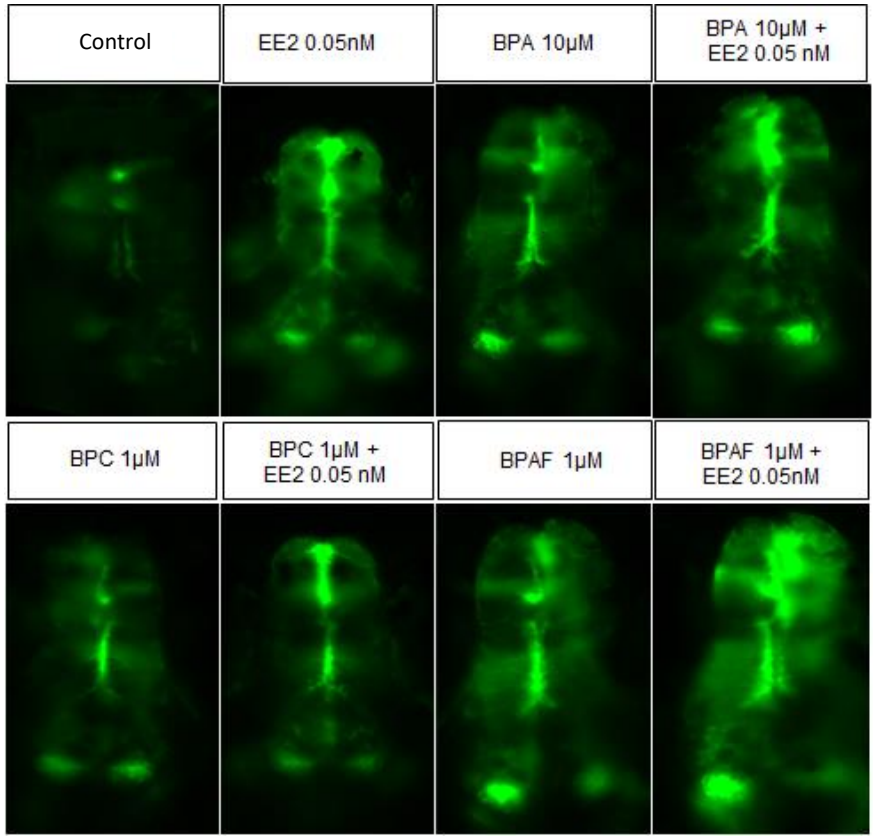
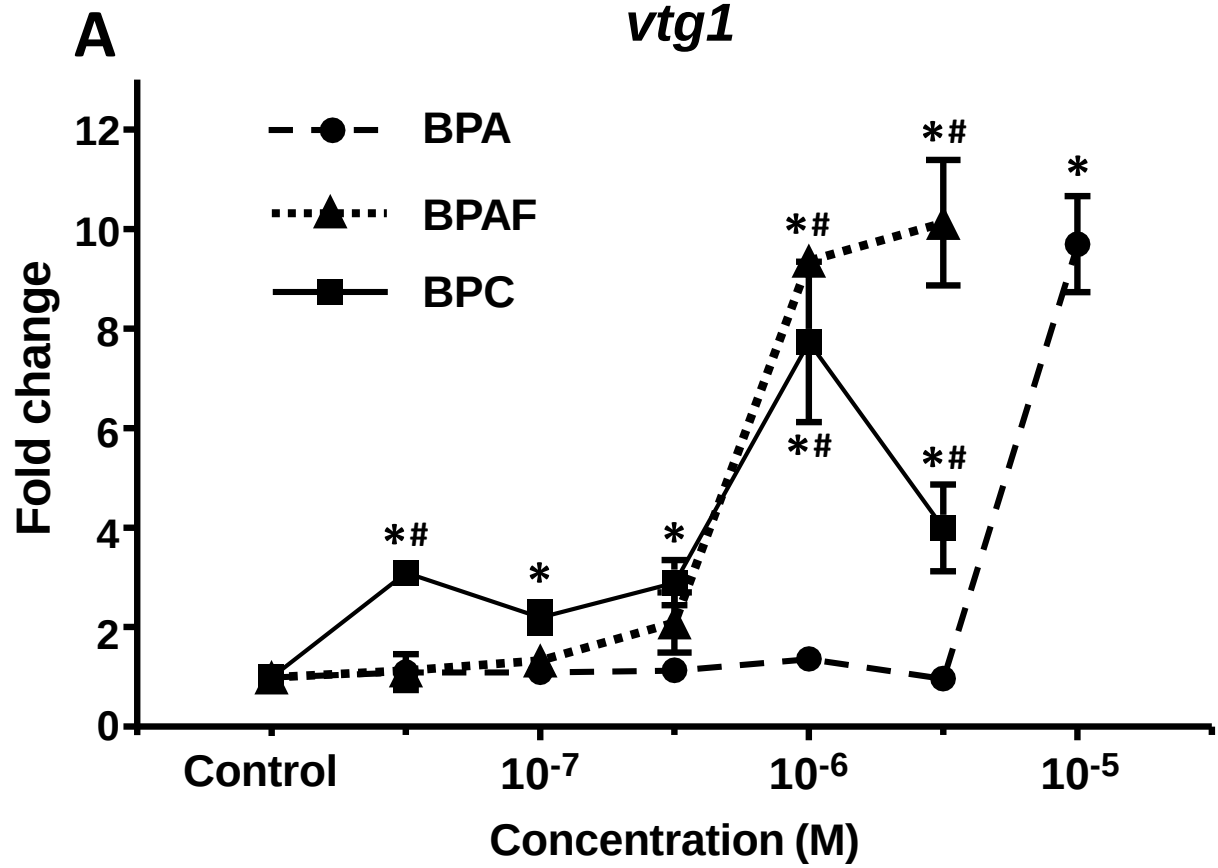

Control
EE2 0.05nM
BPA 10µM
BPA 10µM + EE2 0.05 nM
BPC 1µM
BPC 1µM + EE2 0.05 nM
BPAF 1µM
BPAF 1µM + EE2 0.05nM

Figure 5


A
vtg1
BPA
BPAF
BPC
Fold change
12
10
8
6
4
2
0
*#
*
*
*#
*#
*#
*
Control
10-7
10-6
10-5
Concentration (M)

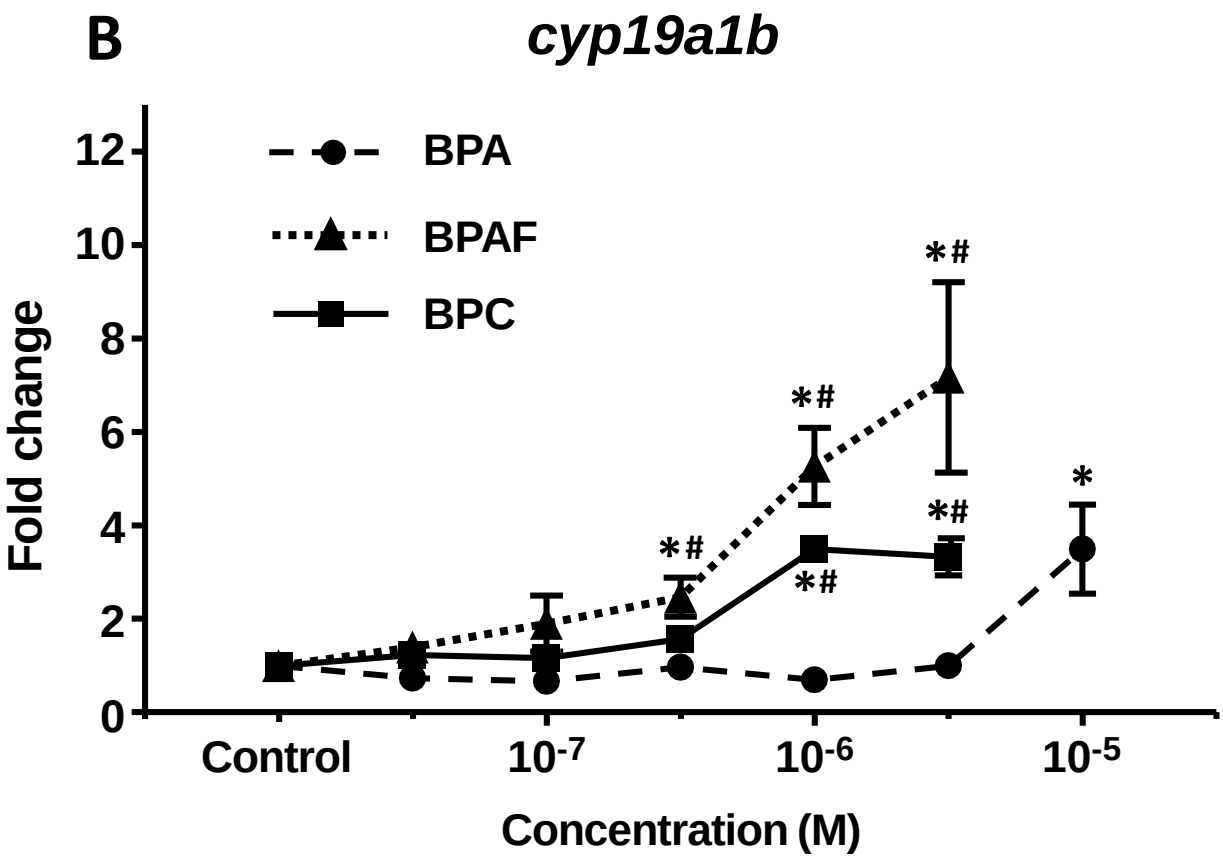

B
cyp19a1b
BPA
BPAF
BPC
Fold change
12
10
8
6
4
2
0
*#
*#
*#
*#
*#
*
Control
10-7
10-6
10-5
Concentration (M)

Figure 6

Table 1. Effective concentration (EC50 in nM), IC50 in nM), relative estrogenic potency (REP) and maximal activity (%) of 17β-E2, 16α-LE2, BPA and the bisphenol analogues on the zfERs.

	zfERα		zfERβ1		zfERβ2	
Basal activity	18.8 ± 0.3		16.5 ± 0.7		20.2 ± 0.6	
Agonism						
Ligand	EC50 (REP)	Maximal activity	EC50	Maximal activity	EC50	Maximal activity
E2	0.08 ± 0.03 (1)	100	0.04 ± 0.01 (1)	100	0.12 ± 0.03 (1)	100
16α-LE2	0.65 ± 0.1 ($1.2\ 10^{-1}$)	100	271 ± 45 ($1.5\ 10^{-4}$)	100	69.5 ± 17.5 ($1.7\ 10^{-3}$)	100
BPA	469 ± 42 ($1.7\ 10^{-4}$)	79	3326 ± 381 ($1.2\ 10^{-5}$)	53.1	2800 ± 562 ($4.3\ 10^{-5}$)	56.1
BPAF	84 ± 27 ($9.5\ 10^{-4}$)	89.3	758 ± 131 ($5.3\ 10^{-5}$)	89.6	897 ± 268 ($1.3\ 10^{-4}$)	79.6
BPC	25 ± 7.5 ($3.2\ 10^{-3}$)	31	316 ± 110 ($1.3\ 10^{-4}$)	36.1	167 ± 72 ($7.2\ 10^{-4}$)	58.5
Antagonism						
	IC50		IC50		IC50	
BPC	46 ± 6.4		160 ± 42		136 ± 75	

Values are the mean ± standard deviations from three separate experiments. Maximal activity is expressed as percentage of luciferase activity induced by 10 nM E2. REPs were determined as the ratio of E2 EC50 to that of bisphenol.

Table 2. Dissociation constant (Kd) values for E2, 16α-LE2, BPA, BPAF and BPC on the zfERs. Values are the mean ± standard deviations from three separate experiments.

	zfERα)	zfERβ1	zfERβ2
Ligands	Kd (nM)	Kd (nM)	Kd (nM)
E2	2.6 ± 0.6	1.1 ± 0.2	2.6 ± 0.7
16α-LE2	9.4 ± 0.9	1109 ± 126	351 ± 44
BPA	1245 ± 265	10040 ± 2919	6884 ± 1270
BPAF	428 ± 85	1023 ± 184	1993 ± 628
BPC	78 ± 16	678 ± 124	402 ± 89