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Bisphenol-A, and Three Related Alkylphenols Exert Rapid Estrogenic Actions on Zebrafish (*Danio rerio*) Oocytes to Maintain Meiotic Arrest

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Bisphenol A, and Three Related Alkylphenols Exert Rapid Estrogenic Actions on Zebrafish (*Danio rerio*) Oocytes to Maintain Meiotic Arrest

by

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Dedication

This work is dedicated to my family, both related and otherwise. Thanks to my mom for always exposing me to the cool things science can do and for having my back whenever I needed it. I'd like to thank my "scientist" father for helping me see the bright side of things and keep my head up. Thanks to Mario for all the support and moving to "an island in Texas?" for me. Thanks to my extended village family for always helping me, taking me to grad school interviews and providing tons of advice whenever I needed it. To all my mentors in undergraduate and at the University of Tennessee you have helped me grow as a scientist and a student this would not be possible without you.

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Abstract

Bisphenol A, and Three Related Alkylphenols Exert Rapid Estrogenic Actions on Zebrafish (*Danio rerio*) Oocytes to Maintain Meiotic Arrest

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The University of Texas at Austin, 2014

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Bisphenol A (BPA) is an alkylphenol compound used in plastic manufacturing, which enters the aquatic environment through wastewater treatment plants and landfill leachates. Exposure of fish to BPA results in developmental defects, decreased hatching, and increased occurrence of the egg yolk precursor protein vitellogenin in male fish. BPA is an estrogenic like compound (xenoestrogen) that can bind and activate the nuclear estrogen receptors, ERα and ERβ, causing changes in gene transcription (genomic mechanism). However, the ability of BPA to activate rapid estrogen signaling and to disrupt nongenomic physiological responses to estrogens is not known. One important nongenomic estrogen action in fish is to maintain meiotic arrest of oocytes and to prevent precocious oocyte maturation through activation of the transmembrane G-protein coupled estrogen receptor (Gper). Binding of estrogens to Gper on zebrafish oocytes results in rapid activation of epidermal growth factor receptor (Egfr) signaling and subsequent Mapk3/1 activation. We show here that BPA and three structurally-related chemicals, tetrachlorobisphenol A, tetrabromobisphenol A and nonylphenol, can mimic estrogen by

activating this mechanism of meiotic arrest through Gper in zebrafish (*Danio rerio*). BPA bound to zebrafish Gper and inhibited spontaneous oocyte maturation (OM) of denuded oocytes. Treatment of oocytes with Actinomycin D did not block the effects of BPA, suggesting that this inhibition of OM is through a nongenomic mechanism. Incubation of oocytes with a selective GPER antibody and the specific GPER antagonist G-15 blocked the effects of BPA on OM, further suggesting that BPA inhibition of OM is through its interaction with the receptor. Various inhibitors of the EGFR pathway were utilized to determine if the inhibition of OM by BPA is mediated through this mechanism. BPA activation of the Egfr pathway resulted in Mapk3/1 (also known as Erk 1/2) phosphorylation. The results show that BPA disrupts oocyte maturation through a novel mechanism involving activation of a Gper/Egfr/Mapk3/1 pathway with potential adverse impacts on reproductive success.

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CHAPTER 1

INTRODUCTION

1.A. TELEOST OOGENESIS

The teleost fish reproductive cycle is regulated by hormones secreted by the hypothalamus-pituitary-gonad axis. Environmental stimuli, such as water temperature and day length, are detected by the sensory organs and interpreted by the hypothalamus (Thomas, 2008). After integration of these signals, the hypothalamus releases gonadotropin releasing hormone, which then stimulates the pituitary to release either follicle stimulating hormone or luteinizing hormone (LH). These two hormones then bind to specific receptors on the granulosa and theca cells to stimulate the various stages of oogenesis.

The stages of teleost oogenesis can be characterized as pre-vitellogenic, vitellogenic, oocyte maturation, and ovulation (Patiño and Sullivan, 2002). The oocyte is surrounded by follicle cells that are composed of two cell types: the inner layer of granulosa cells surrounded by an outer layer of theca cells. In response to gonadotropins the theca cells produce testosterone, which is aromatized by the granulosa cells to estradiol-17β (estradiol) (Kagawa et al., 1982). Estradiol regulates the production of the hepatic yolk precursor protein, vitellogenin, which accumulates in the oocytes resulting in a dramatic increase in their size. During the vitellogenic stage meiosis is arrested in prophase 1 (Khan and Thomas, 1999). A surge in the secretion of LH, a gonadotropin, from the pituitary induces the resumption of meiosis by stimulating the production of maturation inducing hormone (MIH) by ovarian follicle cells (17,20β-dihydroxy-4-pregnen-one in zebrafish). The MIH in turn acts through a nongenomic mechanism to initiate the resumption of meiosis by binding to a membrane progestin receptor (mPRα)

on the oocyte plasma membrane (Zhu et al., 2003; Thomas, 2012). This in turn activates signaling pathways resulting in a decrease in intracellular cAMP levels and activation of maturation promoting factor, resulting in completion of oocyte maturation (Nagahama and Yamashita, 2008).

1.B GENOMIC AND NONGENOMIC ESTROGEN SIGNALING

The classic mechanism of steroid action on target cells involves diffusion of steroids through the cell membrane, their binding to an intracellular receptor belonging to the nuclear receptor superfamily, translocation of the receptor to the nucleus and its binding to the hormone response elements on genes, causing changes in their rates of transcription. Endogenous estrogens regulate the production of numerous proteins needed for oocyte growth, such as the yolk precursor protein vitellogenin, through this genomic mechanism (Masui, 1985).

Estrogens also cause rapid nongenomic signaling through activation of nuclear receptors in extra-nuclear locations and also through the novel 7-transmembrane (7TM) G-protein coupled estrogen receptor, GPER (GPER is the mammalian notation while Gper is the fish notation). Binding of estrogens to GPER results in rapid activation of second messengers and cellular responses that are nongenomic (Filardo et al., 2000; Thomas et al., 2005), through mechanisms similar to those observed with other steroid hormones and their membrane-bound receptors (Revelli et al., 1998). GPER is coupled to a stimulatory G-protein and has high affinity, limited-capacity binding sites for estradiol (Revankar et al., 2005; Thomas et al., 2005). Using the human breast cancer cell line SKBR3, it was observed that estradiol activation of GPER resulted in rapid activation of both adenylyl cyclase and Mapk3/1 (Erk 1/2) (Filardo et al., 2000). GPER,

like other 7TM G-proteins, is coupled to a heterotrimeric G-protein consisting of αβγ subunits, which dissociate from the receptor into Gα and Gβγ upon ligand activation (Clapham and Neer, 1997). Activation of GPER by estrogen results in activation of two separate intracellular signaling pathways. The Gα subunit causes an increase in membrane bound adenylyl cyclase activity resulting in a rise in cAMP levels (Filardo et al., 2002). Activation of the Gβγ subunit results in Erk 1/2 (Mapk3/1) phosphorylation and requires a release of heparin bound EGF, which led to the hypothesis that GPER also transactivates EGFR (Filardo et al., 2000; Peyton and Thomas, 2011). The epidermal growth factor receptors are a family of tyrosine kinase receptors, which are responsible for cellular functions by activating intracellular signaling cascades (Oda et al., 2005). Epidermal growth factor receptors have been studied extensively in cancer research due to the unregulated cell growth which occurs when the intracellular signaling pathways remain active, or when the receptor expression is upregulated (Normanno et al., 2006). Estrogen signaling through GPER results in activation of both the α-subunit-dependent cAMP signaling pathway, and the βγ-dependent EGFR signaling pathway. These discoveries have provided insights into breast cancer progression via a novel mechanism of estrogen signaling in breast cancer cells through GPER.

In the teleost oocytes Gper plays an important role in the regulation of meiotic maturation. Treatment of oocytes with estradiol inhibits meiotic maturation and causes a rise in adenylyl cyclase and subsequent upregulation of cAMP (Pang and Thomas, 2010). Treatment of oocytes with Gper anti-sense oligonucleotides and the transcription inhibitor Actinomycin D, demonstrated that the regulation of zebrafish oocyte maturation is mediated through Gper and is a nongenomic mechanism (Pang and Thomas, 2010). Egfr mRNA was detected in denuded zebrafish oocytes and treatment with EGF caused an increase in the Egfr mRNA expression (Peyton and Thomas, 2011). The presence of

Egfr on the oocyte membrane suggested that the receptor may play a role in estrogen signaling. It was confirmed that estrogen activation of Gper results in Erk 1/2 phosphorylation and is required for inhibition of meiotic maturation (Peyton and Thomas, 2011).

2. BISPHENOL A AS A XENOESTROGEN

Estrogenic actions of BPA were first identified over 70 years ago (Dodds and Lawson, 1938), but the mechanisms by which BPA and other environmental estrogens (xenoestrogens) exert their effects through nongenomic mechanisms, have not been investigated in detail. Environmental estrogen exposure has been correlated with endocrine disruption in addition to reproductive and developmental abnormalities of both aquatic and terrestrial wildlife species (Colborn et al., 1993). The presence of increased levels of the egg yolk precursor protein vitellogenin in both freshwater and marine male fish has been observed after the exposure to wastewater effluent containing weakly estrogenic chemicals (Jobling and Sumpter, 1993). Exposure of zebrafish embryos to BPA increased expression of vitellogenin vtg1 mRNA, increased aromatase expression, and induced physical developmental defects (Kishida et al., 2001; Duan et al., 2008; Chung et al., 2011; Chow et al., 2013). BPA is present in household products and has been detected in wastewater effluents (Colborn et al., 1993). Field surveys from around the world have also detected BPA in both urban and rural waterways and in the marine environment, suggesting that BPA can exert endocrine disrupting effects even at great distances from the source of pollution (Bolz et al., 2001; Kawahata et al., 2004; Ribeiro et al., 2009; Zhang et al., 2009). The two primary sources of environmental BPA are from wastewater treatment plants and landfill leachates (Coors et al., 2003; C.E.P.A.,

2008; Zhang et al., 2009). In a study of 85 streams and rivers in the United States, the U.S.E.P.A. detected BPA over the range of 0.14-12.0µg/L (.61-52.6 nM) (U.S.E.P.A, 2010).

In an aerobic environment BPA degrades in 2-8 days, while in an anaerobic environment, such as in the ocean sediment, degradation is much slower and occurs over a time period of approximately four months (Kang and Kondo, 2002). The NOAEL (no observed adverse effect level) for BPA is currently at 50µg/kg/day in humans, but there is evidence to suggest that low dose exposures below this threshold could also be harmful (vom Saal and Hughes, 2005; U.S.E.P.A, 2010). BPA has been shown to bind to the human membrane estrogen receptor, GPER, with a relative binding affinity of 2-3% to that of estradiol (RBA 2.83) (Thomas and Dong, 2006), which is significantly higher than its affinity for ERα and ERβ (RBA 0.008-1.2) (Blair et al., 2000; Mueller et al, 2003). The relatively high binding affinity of BPA for GPER observed in competitive binding assays suggests it potentially can interfere with endogenous estrogen signaling through this receptor (Thomas and Dong, 2006). BPA has been shown to mimic the actions of estradiol (E2) in the upregulation of cAMP production in mammalian cells transfected with GPER, presumably via activation of membrane adenylyl cyclase through the α-subunit of the stimulatory G protein coupled to GPER (Thomas and Dong, 2006). A similar elevation of cAMP occurs through Gper during estrogen inhibition of OM in zebrafish (Pang and Thomas, 2010). This mechanism has recently been shown to be nongenomic, with no involvement of the nuclear estrogen receptors ERα and ERβ (Pang and Thomas, 2010; Peyton and Thomas, 2011). Interestingly, preliminary evidence suggests that BPA mimics the inhibitory action of E2 on OM in zebrafish and that it involves upregulation of cAMP production in zebrafish oocytes (Peyton & Thomas, unpubl.).

Oocyte maturation is a critical physiological function in teleosts and its susceptibility to inhibition by BPA and the mechanism of endocrine disruption have not been fully explored. Maintenance of meiotic arrest by estradiol through Gper in zebrafish oocytes has recently been shown to also involve transactivation of Egfr and activation of downstream Erk 1/2 signaling (Peyton and Thomas, 2011). Preliminary evidence obtained by Peyton and Thomas, showing that BPA activates Egfr signaling, provides the scientific background for this current study to further explore the effects of BPA and other xenoestrogens on the Egfr mechanism of oocyte meiotic arrest.

3. HYPOTHESIS AND GOALS

The purpose of this study is to investigate whether BPA exerts an estrogenic action on zebrafish oocytes (*Danio rerio*) to inhibit OM and if this occurs through Gper and involves the Egfr/Mapk3/1 signaling pathway. Preliminary research shows that BPA can bind human GPER and cause a rise in intracellular adenylyl cyclase. My preliminary research shows that BPA can inhibit zebrafish oocyte maturation at a similar concentration to that observed with estradiol. This research tests the hypothesis that BPA and structurally-related xenoestrogens activate Gper, mimicking the inhibitory effects of estradiol on zebrafish oocyte maturation. The sub-hypotheses that are investigated to address the above hypothesis are 1) BPA binds to zebrafish Gper and inhibits OM through a nongenomic mechanism; 2) BPA transactivates Egfr and activates Mapk3/1; and 3) three structurally-related chemicals can also bind Gper and inhibit OM.

4. APPROPRIATENESS OF MODEL SPECIES

Zebrafish kept at optimal conditions spawn daily and their ovaries contain oocytes that are asynchronous (present at various stages of maturation). At the height of spawning each fish may produce clutches of 500 eggs or more, which are large in size and transparent. The fully-grown immature eggs used for this study are easily visible under a light microscope and range in size from 600-680μm. Furthermore, the maturation process from meiotic arrest to spawning is hormonally controlled, providing a model for the role of xenoestrogen interference of this steroid controlled pathway. Zebrafish are easily available from commercial hatcheries, and can be bred successfully in the laboratory. They are small in size and relatively easy to maintain in a laboratory setting making them a useful model species for reproductive research.

CHAPTER 2

INTRODUCTION

Thomas and Dong demonstrated that the estrogenic environmental contaminants (xenoestrogens) o,p'-DDE and polychlorinated biphenyls are agonists of human GPER, suggesting an alternative mode of endocrine disruption to that mediated through the nuclear estrogen receptors ERα and ERβ (Thomas and Dong, 2006). The alkylphenol BPA was characterized as a xenoestrogen over 70 years ago and is currently found in the environment in large quantities due to its use in plastic goods (Dodds and Lawson, 1938; C.E.P.A., 2008). E.P.A. sampling studies of streams and rivers have reported BPA at concentrations ranging from 0.14-12.0µg/L (.61-52.6 nM) and biomonitoring studies have detected BPA in human blood at an average level of 4.4nM (U.S.E.P.A, 2010; Vandenberg et al., 2010). Exposure of fish to BPA has been correlated with decreased hatching success (Duan et al., 2008), developmental defects (Duan et al., 2008), increased vitellogenin levels (Chow et al., 2013), and increased brain aromatase expression (Kishida et al., 2001; Chung et al., 2011). While most estrogenic assays focus on nuclear estrogen receptor activation (ERα and ERβ), there is a growing body of evidence suggesting that xenoestrogens may be more potent at activating estrogen receptors through nongenomic mechanisms than through the classic genomic pathway (Thomas and Dong, 2006; Watson et al., 2010). BPA has a higher relative binding affinity for the human membrane estrogen receptor, GPER, than for the nuclear estrogen receptors (Thomas and Dong, 2006). In *in vitro* models of nongenomic signaling, both BPA and nonylphenol can induce calcium (Ca++) influx, prolactin secretion, and Mapk3/1 (Erk 1/2) phosphorylation; however, the mechanisms behind these effects have not been investigated in detail (Wozniak et al., 2005; Kochukov et al., 2009).

The aim of this study was to investigate the possible estrogenic effects of the xenoestrogen BPA mediated through the membrane estrogen receptor Gper in a well-established model of estrogen action through this receptor, the maintenance of oocyte meiotic arrest in zebrafish (Pang et al., 2008; Pang and Thomas, 2009; 2010; Peyton and Thomas, 2011). The effects of BPA on Gper activation and disruption of estrogen regulation of OM in zebrafish was investigated using an *in vitro* OM bioassay. The binding of BPA to zebrafish Gper was assessed with a single point binding assay using zebrafish Gper-transfected HEK293 cells. The potential mechanism of BPA inhibition of OM through activation of an Egfr/Mapk3/1 pathway was investigated. Finally the possible estrogenic actions of tetrachlorobisphenol A, tetrabromobisphenol A, and nonylphenol were also investigated to determine if these closely-related compounds have similar nongenomic estrogenic activities.

MATERIALS AND METHODS

Chemicals:

All chemicals were purchased from Sigma Aldrich unless otherwise stated. AG825 and Ilomastat were purchased from Enzo Life Sciences and U0126 was purchased from Cell Signaling Technology, Inc. Bisphenol A was purchased from City Chemical, and tetrachlorobisphenol A (TCBPA) was purchased from TCI America. 4-nonylphenol (nonylphenol, NP) was obtained from Huntsman Corporation (Port Neches, TX). The specific GPER antagonist, G-15, was a gift from Dr. Eric Prossnitz (University of New Mexico Health Science Center, Albuquerque, NM). The tracer used in the estrogen membrane binding assay, [2,4,6,7-3H] estradiol-17β (84Ci/mmol) ([3H]-E2),

was purchased from Perkin Elmer. The selective antibiotic G418 (geneticin) was purchased from Invitrogen and Actinomycin D was purchased from Biovision.

Animal care: All fish were kept at the University of Texas Marine Science Institute and all animal care and use protocols were approved by the University of Texas at Austin Animal Care and Use Committee (IACUC). Adult zebrafish (*Danio rerio*) were purchased from Freshwater Fish (Lewisville, TX) or Seagrest Farms and maintained on a 14L:10D cycle in 10 gallon tanks with recirculating water. Salinity was maintained at $0.5^{o}/_{oo}$ and the temperature at 28°C. Fish were fed commercial brine shrimp flakes twice daily and live brine shrimp to promote robust oocyte production. After a one week acclimation period the fish were used for oocyte maturation assays.

Zebrafish oocyte maturation bioassay:

a. Harvesting oocytes

For each assay 10-15 gravid females were sacrificed humanely using tricaine methanesulfonate (MS222) at a concentration of 0.01% (Pang and Thomas, 2010). Ovarian follicles were removed and placed in 60% Leibovitz L-15 (L-15) media at 22-24˚C in a sterile plastic culture plate. The ovarian tissue was divided into small fragments using fine forceps and scalpel blades under a binocular microscope. The follicle-enclosed oocytes were removed from the ovarian tissue by pipetting the fragments approximately 50 times with a smooth Pasteur pipette. The follicle enclosed oocytes were then washed three times with L-15 media prior to assay or further treatments.

b. Removal of follicle layers

Treatment of oocytes with the enzyme collagenase at a concentration of 50µg/mL for 45 minutes was successful in removing the follicle layer of cells as shown by DAPI staining (Pang and Thomas, 2010; Peyton and Thomas, 2011). The collagenase was then

removed by washing the oocytes three times in L-15 media. Removal of the follicle cells allowed the effects of the treatments to be investigated in the absence of endogenous estrogen.

c. In vitro OM bioassay

Defolliculated oocytes greater than 450μm were selected and placed in a 24-well plate with 1mL of 22-24˚C L-15 media. Each well contained 20-25 oocytes, and treatments were added after all the oocytes were sorted into their wells. The treatments were dissolved in ethanol, DMSO or methanol and the total volume of each solvent did not exceed 1μL. The vehicle control was ethanol unless otherwise stated. The plate was then placed in a 24˚C incubator and observed at the desired time points, between 3-6 hours. Under a dissecting microscope immature oocytes appear black and are not transparent. The oocytes were counted as mature when the germinal vesicle began to disappear and the oocytes become more transparent (see Peyton and Thomas, 2011 for photograph). Recorded time points were chosen based on the level of spontaneous maturation observed from the vehicle control. In most cases untreated denuded oocytes reach 30% maturation within three hours. However, the level of spontaneous maturation varies based on the batch of zebrafish; therefore, all observations were made at 3 hours unless otherwise stated. All treatments were replicated three times (n=3) and all experiments were repeated at least three times. The percent germinal vesicle breakdown (% GVBD) was calculated as number of mature oocytes divided by the total, multiplied by 100, (mature/total)*100. Graph Pad Prism software was used to graphically represent the data. Using the software a one-way ANOVA was performed and the non-parametric Bonferroni post-hoc test was used.

d. Treatments

To ensure that oocytes were maturationally competent the maturation inducing hormone for zebrafish, DHP-17,20β-dihydroxy-4-pregen-3-one, was used at a concentration of 5nM for all assays. The inhibitory effect of BPA was investigated in the presence of various Egfr signaling pathway inhibitors. The inhibitors were prepared and administered as described by Peyton and Thomas (2011). Actinomycin D was used at a concentration of 0.1μg/mL (Peyton and Thomas, 2011). The selective antagonist G15 was used at a concentration of 100nM (Peyton and Thomas, 2011). The SRC inhibitor PP2 and the matrix metalloproteinase inhibitor (MMP) Ilomastat (GM6001) were used at a concentration of 10μM (Zheng et al., 2007; Quinn et al., 2009). The EGFR (ErbB1) inhibitor AG825 was used at a concentration of 50μM (Filardo et al., 2000). The Map2K1/2 inhibitor U0126 was tested at a concentration of 50μM (Mood et al., 2004). The polyclonal croaker Gper antibody raised in rabbits and rabbit IgG were used at a concentration of 1:300 (Pang and Thomas, 2010).

Culture of Gper-transfected cells and membrane preparation:

HEK293 cells stably transfected with zebrafish Gper mRNA following the same procedures as those used for human GPER (Thomas et al., 2005), were used for the binding study. Cells were cultured in DMEM/Ham's F-12 media without phenol red, supplemented with 5% FBS and 750ug/mL G418. After 7-8 days of growth cells were harvested by washing three times with ice-cold phosphate buffered saline solution (PBS), followed by the addition of 5mL ice-cold HAED (HEPES 25mM, NaCl 10mM, Dithioerythritol 1mM, EDTA 1mM) and removal of the cells with a cell scraper. Whole cells were either frozen at -80˚C or used immediately. The cell suspension was centrifuged for 5 minutes at 1500 x g. The cells were resuspended in 4mL HAED with 0.1% protease inhibitor and then centrifuged again at 1500 x g for 5 minutes. The cells

were resuspended in 4mL HAED and subsequently sonicated for 7 seconds. The cell homogenate was then centrifuged for 7 minutes at 1000 x g and the supernatant collected. The supernatant was centrifuged at 20,000 x g for 25 minutes and the pellet was resuspended in HAED to the desired concentration of 1-1.5mg/mL of cell membrane protein.

Estrogen receptor competitive binding assay:

The assay was conducted as previously described (Thomas et al., 2005) with minor modifications. Reaction vials contained 4nM of [3H]-E2 (dissolved in ethanol and prepared in HAED buffer), steroid competitors at a concentration of 1μM or 0.1μM, ethanol and 0.5-0.7mg/mL membrane preparation. The total volume of the reaction was 303μL which included 150μL of prepared membrane, 150μL of tracer and 3μL of cold competitor dissolved in ethanol (ethanol did not exceed 1% of the total volume, thereby not affecting [3H]-E2 binding). Membrane fractions were incubated with [3H]-E2 and competitors for 30 minutes at 4°C and reactions were stopped by filtration through Whatman GF/B filters. The filters were transferred to scintillation vials, scintillation cocktail was added, and the radioactivity was measured by scintillation counting. The displacement of [3H]-E2 from the plasma membrane fractions by the competitors was expressed as a percentage of the maximum E2 specific binding. All reactions were run in triplicate.

Erk 1/2 activation quantification by Western blot analysis:

Zebrafish ovarian follicles were harvested and prepared as described for the oocyte maturation bioassay. After defolliculation the oocytes were transferred to a 24-well plate with 1mL of L-15 media at 22-24°C. The oocytes were incubated with the various treatments for 15 minutes at 24˚C, and the reaction was stopped by placing the

plate in an ice bath. The oocytes were removed and briefly centrifuged (5 seconds at 1000 x g) and the L-15 media discarded. The oocytes were then washed with 1mL ice-cold PBS and centrifuged briefly (5 seconds at 1000 x g). The excess PBS was removed and the oocytes were then stored overnight at -80˚C. The following day the oocyte lysates were prepared as described previously (Peyton and Thomas, 2011). 100uL of ice cold RIPA buffer with 0.1% protease inhibitor was added to the thawed oocytes. The oocytes were then homogenized for one minute using a glass handheld homogenizer on ice. After homogenization the samples were vortexed for 30 minutes at 4˚C. To remove the insoluble material the samples were centrifuged at 15,000 x g for 5 minutes. The supernatant was removed and protein was quantified using the Bradford protein assay and analyzed with the NanoDrop 2000 software (Thermo Scientific). The protein was boiled with reducing buffer for 5 minutes and 15µg protein per lane was electrophoresed on a 10% sodium dodecyl sulfate polyacrylamide gel-electrophoresis (SDS-PAGE) gel. The protein was then transferred for 1 hour at 0˚C to a nitrocellulose membrane (Bio-Rad). Membranes were blocked for one hour at room temperature in blocking buffer (1.5g dry milk, 30mL PBS, 30µL Tween-20). Membranes were then incubated with the primary antibody for phosphorylated Erk- rabbit (phospho-p44/42 Mapk (Erk 1/2) Cell Signaling) and the primary antibody for total Erk-mouse (p44/42 Mapk (Erk 1/2) Cell Signaling) overnight at 4°C. Membranes were washed and then incubated with secondary antibodies, goat anti-rabbit 800nm and goat anti-mouse 680nm for 1.5 hours. The membranes were photographed using the Li-Cor Odyssey imager and protein expression was quantified by measuring densitometry using Image J software from the Public Research Centre Henri Tudor (Luxembourg-Krichberg). The change in protein expression was quantified by comparing relative density of phosphorylated Erk 1/2 compared to total Erk as a loading control.

RESULTS

Effects of BPA on OM

Approximately 25% of the vehicle-treated (1μL ethanol) denuded oocytes had undergone spontaneous GVBD by the end of the 3-hour incubation period (Fig. 1). Treatment with 5nM of the zebrafish MIH dihydroxyprogesterone (DHP) caused a further marked increase in % GVBD within 3 hours (Fig. 1), indicating that the oocytes were maturationally competent. Treatment with E2 100nM, for 3 hours caused a significant decrease in spontaneous OM (~10% GVBD) compared to that in the vehicle-treated group (Fig. 1). These inhibitory effects of E2 on OM were mimicked by BPA over the range of concentrations of 100nM to 10nM, whereas % GVBD after treatment with 5nM BPA was not significantly different from vehicle controls (Fig. 1). These inhibitory effects of BPA on OM, like those of E2, were transitory. The % GVBD was not significantly different from vehicle controls after long term incubation with BPA and E2 (Fig. 2). These results suggest that the impairment of GVBD at 3 hours is not due to a nonspecific toxic action of BPA.

Effects of Actinomycin D on BPA

To determine whether BPA acts via a genomic mechanism to inhibit OM, denuded oocytes were co-treated with BPA and the transcription inhibitor, Actinomycin D (0.1μg/mL). Co-treatment with Actinomycin D did not blunt the inhibitory action of 100nM BPA on OM (Fig. 3), indicating that it is acting through a nongenomic mechanism.

Role of Gper in the inhibition of OM by BPA

The possible role of Gper in mediating the nongenomic estrogen actions of BPA to inhibit spontaneous maturation of denuded oocytes was investigated using a specific Gper antibody. Co-incubation of the specific Gper antibody with BPA completely blocked BPA inhibition of OM, whereas incubation with the control rabbit IgG was ineffective, which suggests BPA acts through Gper to regulate OM (Fig. 4).

The role of Egfr signaling in the inhibition of OM by BPA

The involvement of the Egfr pathway in the inhibitory effects of BPA on OM was investigated using specific inhibitors of different components of the pathway. Incubation of denuded oocytes with 10μM of the selective SRC kinase inhibitor PP2 alone did not significantly alter the % GVBD compared to that of the vehicle controls (Fig. 5). Co-treatment with PP2 attenuated the inhibitory effects of BPA, causing a significant increase in OM compared to that induced by BPA alone (Fig. 5). Similar effects were observed with the MMP inhibitor ilomastat (Fig. 6). Treatment with 10μM ilomastat alone did not significantly alter the % GVBD compared to that of the vehicle controls (Fig. 6). Co-treatment of oocytes with ilomastat blocked the inhibitory effects of BPA on GVBD (Fig. 6). Finally, incubation of oocytes with the selective EGFR inhibitor AG825 50μM alone resulted in a significant increase in % GVBD compared to that of the vehicle controls (Fig. 7). Co-treatment with AG825 also completely blocked the inhibitory effects of BPA and E2 on OM. The combined treatment of AG825 and BPA significantly increased the % GVBD compared to controls (Fig. 7).

Involvement of Mapk3/1 signaling in inhibition of OM by BPA

The role of Mapk3/1 signaling in the effects observed with BPA was examined using the Map2k inhibitor U0126. Treatment with U0126 alone did not significantly alter

the % GVBD compared to controls, whereas co-treatment of U0126 blocked the inhibitory effects of BPA on OM, resulting in a significant increase in the maturation of oocytes compared to that of the BPA treatment (Fig. 8).

The effects of the structurally related alkylphenols on OM

The effects of TBBPA, TCBPA, and NP on maturation of denuded oocytes were investigated over the same range of concentrations as those used for BPA, 5nM-100nM. A comparison of the effects of 100nM E2, BPA, TCBPA, TBBPA, and NP showed that they were all equally effective at this concentration inhibiting spontaneous maturation, significantly decreasing the % GVBD compared to vehicle controls (Fig. 9). TBBPA significantly decreased the % GVBD compared to the vehicle controls over the concentration range of 10nM to 100nM, whereas 5nM did not significantly decrease OM (Fig. 10). The inhibition of OM by these TBBPA concentrations was temporary and no inhibition of OM was observed after a longer-term 5 hour incubation (Fig. 11). This suggests that the inhibition of OM by TBBPA is not due to a nonspecific toxic effect of the compound. NP was effective in inhibiting OM at all concentrations tested, significantly decreasing the % GVBD over the concentration range from 5nM to 100nM (Fig. 12). As observed for the other compounds, the inhibition of OM by NP was temporary, and the % GVBD increased after 5 hours and was not significantly different from vehicle control (Fig. 13). Similarly, TCBPA inhibited spontaneous OM over the entire concentration range, 5nM to 100nM (Fig. 14) and the % GVBD recovered after 5 hours incubation (Fig. 15). To determine a no effect concentration for NP and TCBPA, oocytes were treated with these compounds at 2.5nM and 1nM concentrations (Fig. 16). No effect on % GVBD was observed for these compounds at these low concentrations.

The role of Gper in mediating the inhibitory effects of TCBPA, TBBPA, and NP on OM was investigated using the specific GPER antagonist G-15. Incubation with G-15 alone did not significantly affect the percent GVBD of denuded oocytes, but co-treatment of G-15 with the alkylphenol compounds reversed their inhibitory effects on OM, which were not significantly different from vehicle controls (Fig. 17).

The involvement of Egfr signaling in inhibition of OM by these alkylphenols was investigated using the EGFR inhibitior, AG825. Treatment with AG825 reversed the inhibitory effects of NP, TBBPA, and TCBPA on OM, significantly increasing the % GVBD above control levels (Fig. 18).

Binding of BPA, TCBPA, TBBPA, and NP to zebrafish Gper

A single-point competitive binding assay showed that 1μM BPA, NP, TBBPA, and TCBPA were all effective competitors of [3H]-E2, binding to membranes prepared from cells expressing recombinant zebrafish Gper (Fig. 19). 1μM TCBPA and NP displaced 62% of the bound [3H]-E2 while 1μM TBBPA and BPA displaced 61% and 60% of the E2 tracer, respectively. One tenth this concentration of E2 (100nM) caused a similar displacement of bound [3H]-E2 (62%) suggesting that these alkylphenols have relative binding affinities approximately one tenth that of E2.

Western Blotting: Erk 1/2 activation

Results from previous experiments in this study suggest that BPA activation of Egfr results in activation of the MAPkinase cascade. To confirm this hypothesis the effects of BPA on Erk 1/2 phosphorylation were investigated by Western blot analysis. Oocytes treated for 15 minutes with E2 and EGF showed increased Erk phosphorylation compared to controls. Increasing concentrations of BPA increased Erk phosphorylation in a concentration-dependent manner (Fig. 20).

DISCUSSION

A major finding of this study is that BPA mimics the action of estradiol through Gper via a nongenomic mechanism to activate Egfr at the cell surface of the oocyte, resulting in maintenance of meiotic arrest. One mechanism by which estradiol inhibits oocyte maturation in zebrafish is through upregulation of cAMP production (Pang and Thomas, 2010). Previously, Thomas and Dong (2006) found that BPA can bind to the human GPER and is capable of upregulating intracellular cAMP in breast cancer cells. These findings in human cells suggested that BPA might also interfere with this nongenomic mechanism of estrogen signaling in teleosts resulting in maintenance of oocyte meiotic arrest. In support of this, preliminary research showed that BPA can also induce a rise in cAMP in zebrafish oocytes and can maintain meiotic arrest of oocytes in zebrafish (Peyton and Thomas unpbl.). The results of the current more extensive study confirm that BPA acts via a nongenomic estrogen mechanism through Gper to inhibit OM in zebrafish oocytes. A second mechanism by which estrogen inhibits oocyte maturation is through transactivation of Egfr (Peyton and Thomas, 2011). The activation of both cAMP and EGFR by estrogen is a well-documented mechanism of nongenomic estrogen signaling through GPER (Filardo et al., 2000; Thomas et al., 2005). Recently, Peyton and Thomas (2011) showed that estrogen inhibition of oocyte maturation also requires transactivation of the Egfr complex. Egfr mRNA is present in denuded oocytes and is located on the plasma membrane, suggesting that estradiol acts at the cell surface (Peyton and Thomas, 2011).

The Egfr inhibitors used in the present study were successful at blocking the effects of BPA on oocyte maturation, suggesting that activation of this signaling pathway is critical for mediating the effects of BPA on OM. Each inhibitor used was as equally effective in blocking the effects of BPA as they were in blocking the effects of E2 in the

study by Peyton and Thomas (2011). This study is the first to show that BPA acts through this nongenomic estrogen mechanism to maintain oocyte meiotic arrest in zebrafish. Though the nongenomic effects of this compound have only recently been explored, the genomic effects of BPA are well characterized.

A significant amount of research has been done on the genomic effects of BPA on the oocytes of teleosts and mammals (Bhandari et al., 2014). Human oocytes incubated with BPA show upregulation of nuclear estrogen receptors and multiple genes responsible for DNA repair (Brieno-Enriquez et al., 2012). BPA can also increase expression of vitellogenin mRNA, vtg1, in unfertilized rainbow trout oocytes and fertilized zebrafish embryos (Chow et al, 2013; Aluru et al, 2010). Of the compounds tested, BPA induced the highest change in gene expression compared to TBBPA, endosulfan, heptachlor, and methoxychlor (Chow et al, 2013). These results were all observed at much higher concentrations (100-1000x) than those used in this study. In general, the potencies of these xenoestrogens in mimicking the genomic actions of estradiol are related to their relative binding affinities for nuclear estrogen receptors. Therefore, measurement of the extent of receptor activation using receptor binding methods, which quantify the ligand binding affinity compared to endogenous estrogen, is valuable for predicting the relative potencies of these compounds.

The primary structural requirement for ligand binding to the nuclear estrogen receptor is a phenolic ring, or ring structure containing a hydroxyl group (Blair et al., 2000). In most cases this ring structure is a phenol but rare exceptions to this rule include compounds such as kepone, which have a chlorodecone structure (Blair et al., 2000). BPA binds relatively weakly to nuclear estrogen receptors with a binding affinity of just 0.008-1.2% for ERα and 1.2% for ERβ (Blair et al., 2000; Mueller et al., 2003)*;* yet, it binds with much higher affinity to human GPER with a RBA of 2.83% (Thomas and

Dong, 2006). Removal of one of the phenol groups from BPA in some cases does not reduce nuclear estrogenic activity. Nonylphenol has only one phenol group, yet the estrogenic activity in nuclear ER reporter assays is not hindered (Van den Belt et al., 2004). Nonylphenol also has a higher RBA for human GPER (2.15%) than for the nuclear ERs (0.003% ERα and β combined) (Blair et al., 2000; Thomas and Dong, 2006). In this study, nonylphenol was more effective at inhibiting oocyte maturation and equally as effective at binding to Gper as BPA. The addition of one methyl group in the meta position of the phenol ring had no effect on the binding affinity for the nuclear ERs (Perez et al., 1998). However, the addition of two bromine atoms in the meta position of the phenolic ring caused a significant decrease in the nuclear estrogen response (Meerts et al., 2001). TBBPA has a RBA of 0.013% for ERα, and did not bind to the nuclear ERs in affinity columns (Olsen et al., 2003; Riu et al., 2014). If the bromine atoms are replaced with chlorine, the estrogen response in nuclear ER reporter luciferase assays increases. In the MCF7 breast cancer cell line TCBPA resulted in the highest estrogenic response compared to BPA and TBBPA (Kitamura et al., 2005). The RBA of TCBPA for ERα and ERβ has not been assessed; however, due to the positive estrogen response in genomic assays TCBPA most likely binds to the nERs. In this study TBBPA and TCBPA show similar displacement for [3H]-E2 suggesting that the different Cl or Br substituents does not affect their binding to Gper. It can be inferred from these results and others that bisphenol-like structured compounds may be inherently more effective at binding Gper than nERs, which lends some insight into the structure of the binding pockets of these receptors.

In this study the structurally related alkylphenols, TBBPA, TCBPA, and NP, were one tenth as effective as E2 at displacing [3H]-E2 but equally effective at inhibiting oocyte maturation. In previous studies, E2 inhibited OM at the lowest treatment of 5nM,

suggesting that NP and TCBPA are equally as potent inhibitors of zebrafish OM as E2 at 5nM (Pang and Thomas, 2010). In a similar study using gobies, Hwang et al. (2010) found that NP could inhibit OM at the lowest concentration of 0.45nM and that 43nM caused an increase in the production of endogenous E2. All of the concentrations tested in this study, except for treatments of 5nM or below, resulted in a maximum inhibition of OM. Furthermore, in all of the experiments 100nM of each chemical resulted in inhibition that was not significantly different than 100nM of E2. These results contrast with the receptor binding results that show E2 to be more effective at displacing [3H]-E2. Treatment of oocytes with 10nM E2 also resulted in a maximal response, though 10nM E2 resulted in less than 50% binding to Atlantic croaker Gper (Pang et al., 2008). This suggests that receptor binding data alone are not sufficient to determine a compound's ability to interfere with a cellular signaling pathway, and that the *in vitro* OM assay is probably very sensitive to steroid interference.

The estrogenic action of BPA is most likely very complex. It has been shown that BPA can bind and activate both the nuclear estrogen receptors (ERα and β) and the membrane estrogen receptor (GPER). It is also known that BPA can bind to the orphan nuclear receptor, human estrogen related receptor γ (ERRγ) (Matsushima et al., 2008). Though ERRγ activates estrogen response related genes, 17β-estradiol is not a ligand of ERRγ (Huppunen and Aarnisalo, 2004). BPA can preserve the action of ERRγ allowing gene transcription (Takayanagi et al., 2006). This receptor has also been implicated in many effects that are observed with BPA exposure in both human cells and zebrafish (Saili et al., 2012). In this study, treatment of oocytes with Actinomycin D did not block any effects observed from BPA treatment. This suggests that transcription of genes is not responsible for the changes in maturation observed from BPA exposure. However, the

role of other estrogen receptors, such as ERRγ, in models of endocrine disruption needs to be explored more.

Here we report that BPA acts through Gper to transactivate Egfr, mimicking estrogen's action on this mechanism. This action through Gper is shared by three chemicals that are structurally similar to BPA; TCBPA, TBBPA and NP. Transactivation of Egfr occurs through the Gβγ subunit of Gper and is a conserved mechanism occurring in both mammals and teleosts (Filardo et al., 2000; Filardo et al., 2002; Peyton and Thomas, 2011). Results from this study provide evidence to support the hypothesis that BPA can act through a nongenomic estrogen signaling pathway via Gper and disrupt a critical cellular function, oocyte maturation, in zebrafish.

CHAPTER 3

SUMMARY AND CONCLUSIONS

Xenoestrogens affect the endocrine system in a very unique way, instead of causing damage to cells, xenoestrogens compete with endogenous steroid hormones by binding to membrane and nuclear receptors. This altered receptor binding can influence gene expression and/or nongenomic cellular signaling. As we learn more about the endocrine system it is becoming more apparent that endocrine disruption can occur in many different ways. Non-classical rapid steroid hormone signaling, which does not involve gene transcription, is quickly becoming an area of study for endocrine disruptor research. The finding that compounds such as BPA have a higher binding affinity for the membrane estrogen receptor GPER, than they do the nuclear estrogen receptor, suggests that there is a more multifaceted mechanism of endocrine disruption than previously thought. Some compounds which may fail to activate common genomic estrogen tests, may in fact be potent nongenomic signaling activators. One example is the compound TBBPA. In many studies TBBPA is a very weak nuclear estrogen agonist, fails to activate luciferase assays, and binds to the nuclear ERs with very low affinity. In this assay TBBPA bound to Gper and inhibited oocyte maturation. The next step would be to measure the relative binding affinities of the chemicals tested in this study. A comparison of relative binding affinities of TBBPA, TCBPA, and NP would be valuable to understand more about the structure activity of these compounds.

The complexity of the mechanism of estrogenic activation by xenoestrogens makes any regulatory decisions difficult. In the U.S. nonylphenol is regulated based on its estrogenic activity but the compound BPA is not. However, in Canada BPA is listed as a toxic chemical and is strictly regulated. Results from this study will contribute to the

knowledge of BPA as a xenoestrogen and aid in regulatory decisions about estrogenic chemicals. Also, this study provides the first evidence that BPA can activate the Gper/Egfr/Mapk3/1 pathway of meiotic arrest in zebrafish oocytes at environmentally relevant concentrations. This *in vitro* study provides some insight into a mechanism of estrogen signaling in the zebrafish oocyte and how that mechanism can be disrupted by an exogenous chemical.

Figures

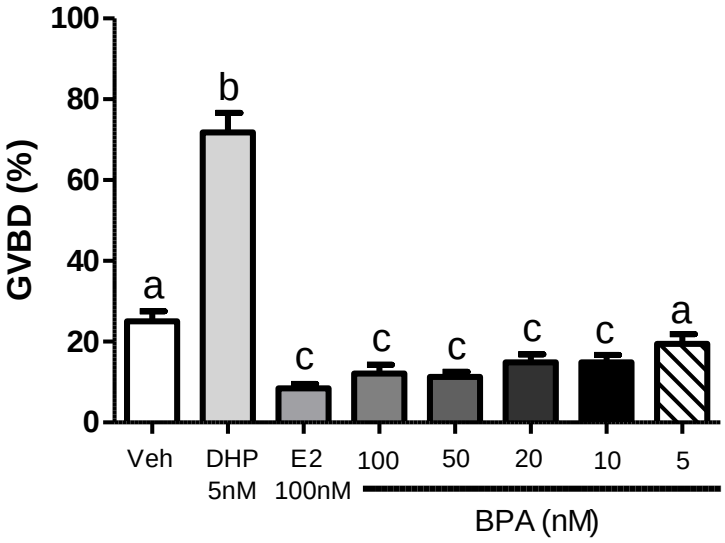


Fig. 1 Effects of BPA over the concentration range of 5nM to 100nM on inhibition of spontaneous maturation of denuded zebrafish oocytes in the *in vitro* GVBD bioassay. Vehicle- control ethanol; DHP- 17,20β-dihydroxy-4-pregen-3-one; E2- Estradiol-17β; BPA-Bisphenol A. Bars denote means ±SEM. Different letters denote significant differences from each other (p <0.05, one-way ANOVA and nonparametric Bonferroni test).

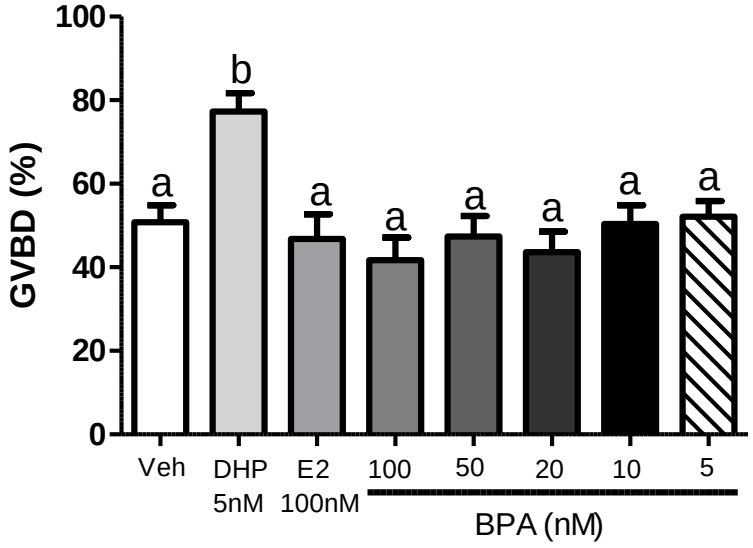


Fig. 2 Effects of BPA over the concentration range of 5nM to 100nM on inhibition of spontaneous maturation of denuded zebrafish oocytes in the *in vitro* GVBD bioassay. Observation made at 5 hours. Vehicle-control ethanol; DHP- 17,20β-dihydroxy-4-pregen-3-one; E2- Estradiol-17β; BPA-Bisphenol A. Bars denote means ±SEM.

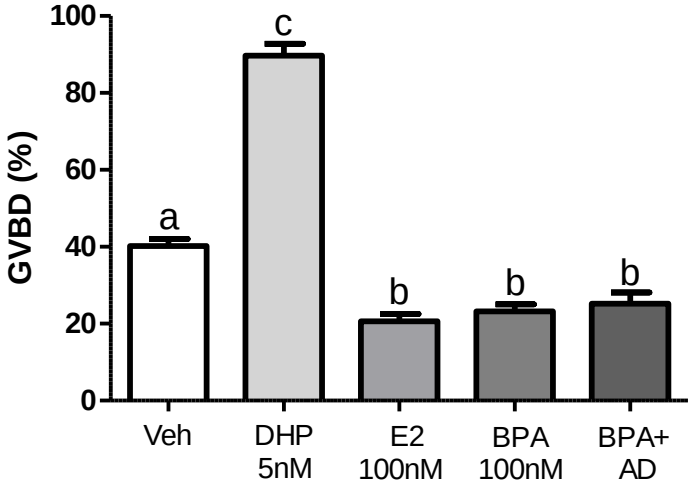


Fig. 3 Effects of the transcription inhibitor Actinomycin D on the inhibitory effects of BPA on spontaneous maturation of denuded zebrafish oocytes in the *in vitro* GVBD bioassay. Vehicle-control DMSO; DHP- 17,20β-dihydroxy-4-pregen-3-one; E2-Estradiol-17β; BPA-Bisphenol A; BPA+AD-100nM BPA with 0.1µg/mL Actinomycin-D. Bars denote means ±SEM. Different letters denote significant differences from each other (p <0.05, one-way ANOVA and nonparametric Bonferroni test).

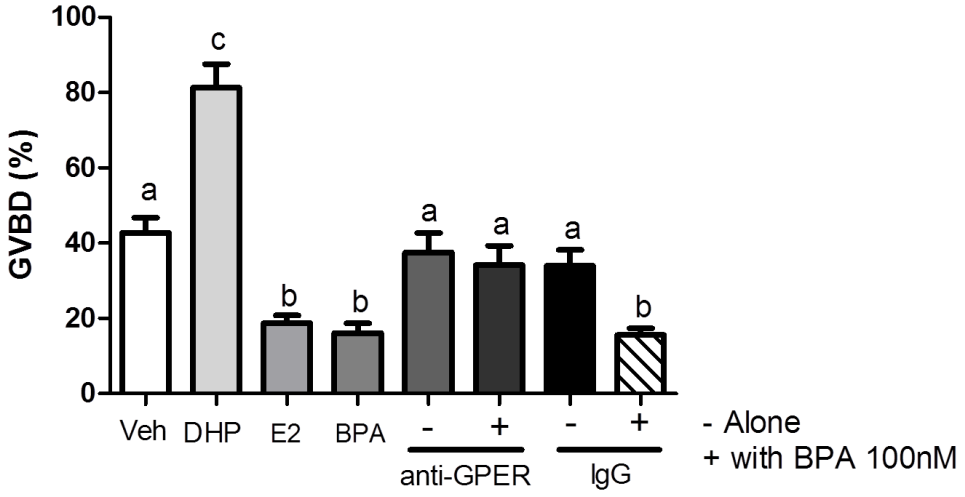


Fig.4 Effects of the specific GPER antibody on the inhibitory effects of BPA on spontaneous maturation of denuded zebrafish oocytes in the *in vitro* GVBD bioassay. Vehicle-control ethanol; DHP- 17,20β-dihydroxy-4-pregen-3-one 5nM; E2- Estradiol-17β 100nM; BPA-Bisphenol A 100nM; anti-GPER+BPA 1:300 GPER antibody with 100nM BPA; IgG- 1:300 rabbit serum. Bars denote means ±SEM. Different letters denote significant differences from each other (p <0.05, one-way ANOVA and nonparametric Bonferroni test).

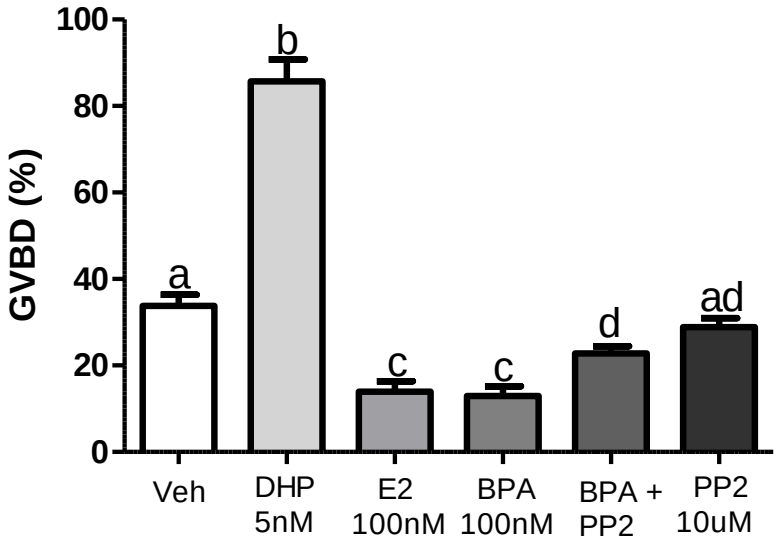


Fig. 5 Effects of the SRC kinase inhibitor PP2 on the inhibitory effects of BPA on spontaneous maturation of denuded zebrafish oocytes in the *in vitro* GVBD bioassay. Vehicle-control ethanol; DHP- 17,20β-dihydroxy-4-pregen-3-one; E2- Estradiol-17β; BPA-Bisphenol A; BPA+PP2-100nM BPA with 10μM PP2; PP2- the SRC kinase inhibitor PP2. Observation made at 4 hours. Bars denote means ±SEM. Different letters denote significant differences from each other (p <0.05, one-way ANOVA and nonparametric Bonferroni test).

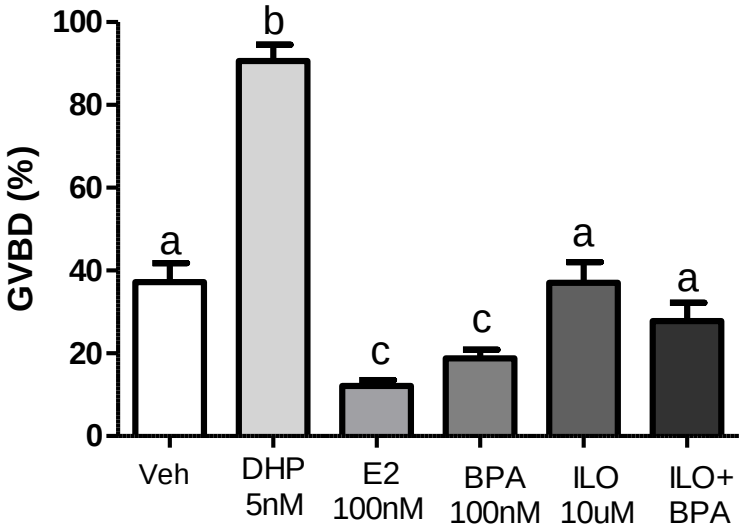


Fig. 6 Effects of the MMP inhibitor Ilomastat on the inhibitory effects of BPA on spontaneous maturation of denuded zebrafish oocytes in the *in vitro* GVBD bioassay. Vehicle-control ethanol; DHP- 17,20β-dihydroxy-4-pregen-3-one; E2- Estradiol-17β; BPA-Bisphenol A; ILO- Ilomastat; BPA+ILO-100nM BPA with 10μM Ilomastat. Bars denote means ±SEM. Different letters denote significant differences from each other (p <0.05, one-way ANOVA and nonparametric Bonferroni test).

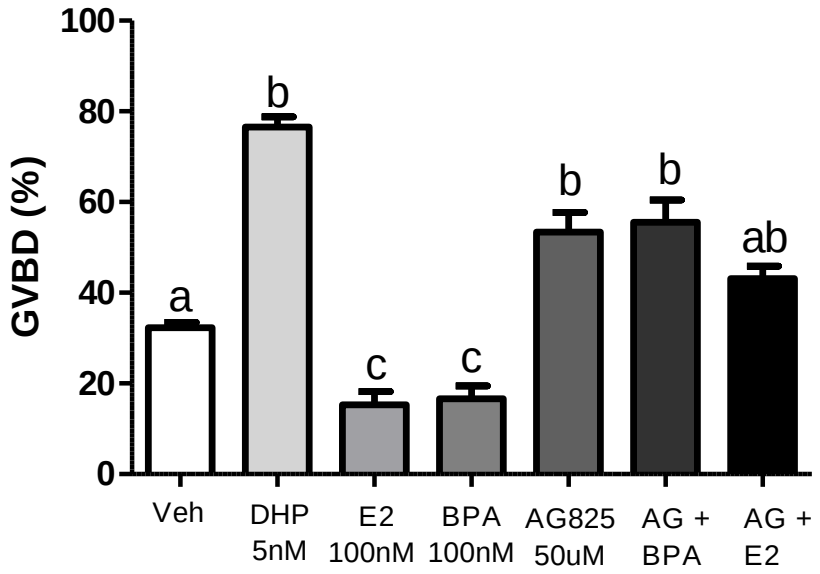


Fig. 7 Effects of the EGFR inhibitor AG825 on the inhibitory effects of BPA on spontaneous maturation of denuded zebrafish oocytes in the *in vitro* GVBD bioassay. Vehicle-control ethanol; DHP- 17,20β-dihydroxy-4-pregen-3-one; E2- Estradiol-17β; BPA-Bisphenol A; AG825-EGFR inhibitor; AG+BPA- 50µM AG825 with 100nM BPA; AG+E2- 50µM AG825 with 100nM estradiol. Bars denote means ±SEM. Different letters denote significant differences from each other (p <0.05, one-way ANOVA and nonparametric Bonferroni test).

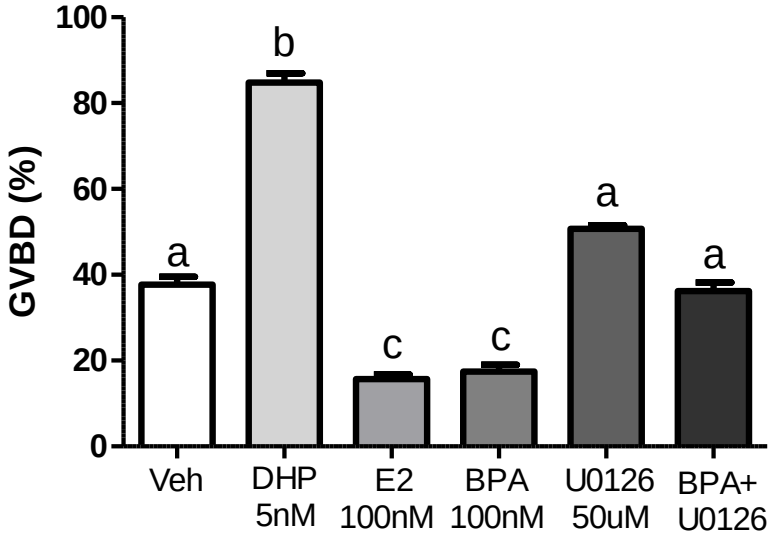


Fig. 8 Effects of the Map2K inhibitor U0126 on the inhibitory effects of BPA on spontaneous maturation of denuded zebrafish oocytes in the *in vitro* GVBD bioassay. Vehicle-control ethanol; DHP- 17,20β-dihydroxy-4-pregen-3-one; E2- Estradiol-17β; BPA-Bisphenol A; U0126 50μM, BPA+U0126- 100nM Bisphenol A with 50μM U0126. Bars denote means ±SEM. Different letters denote significant differences from each other (p <0.05, one-way ANOVA and nonparametric Bonferroni test).

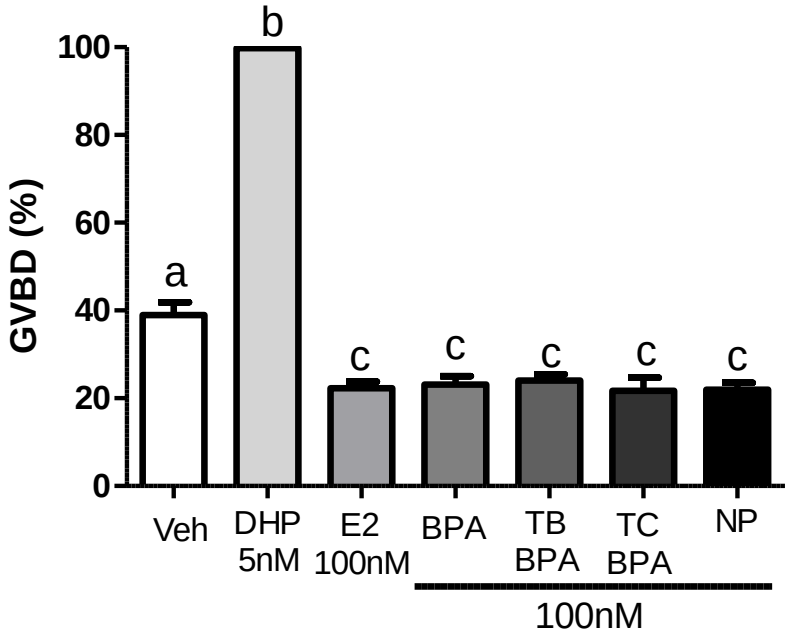


Fig. 9 Effects of the structurally related alkylphenols on spontaneous maturation of denuded zebrafish oocytes in the *in vitro* GVBD bioassay. Vehicle-control ethanol; DHP- 17,20β-dihydroxy-4-pregen-3-one; E2- Estradiol-17β; BPA- 100nM of Bisphenol A; TCBPA- 100nM of tetrachlorobisphenol A; TBBPA- 100nM of tetrabromobisphenol A; NP- 100nM of nonylphenol. Bars denote means ±SEM. Different letters denote significant differences from each other (p <0.05, one-way ANOVA and nonparametric Bonferroni test).

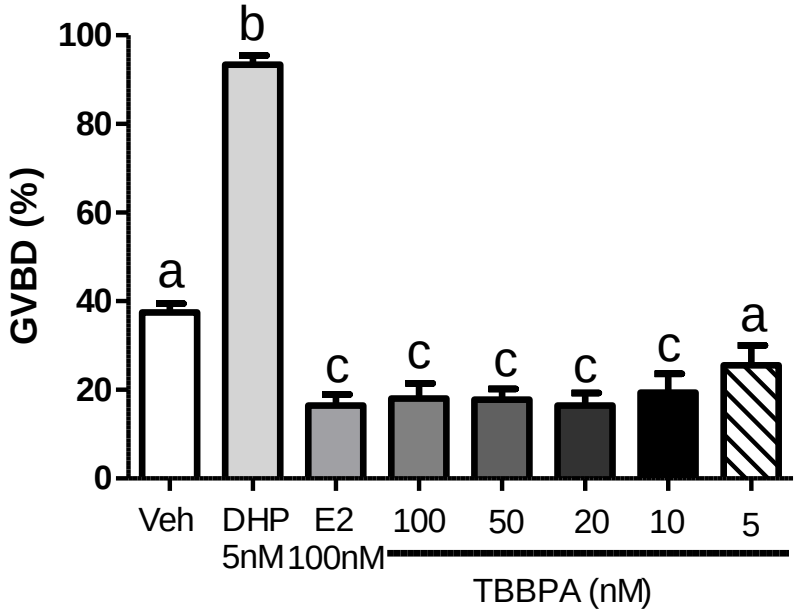


Fig. 10 Effects of various concentrations of tetrabromobisphenol A on spontaneous maturation of denuded zebrafish oocytes in the *in vitro* GVBD bioassay. Vehicle-control ethanol; DHP- 17,20β-dihydroxy-4-pregen-3-one; E2- Estradiol-17β; TBBPA-tetrabromobisphenol A. Bars denote means ±SEM. Different letters denote significant differences from each other (p <0.05, one-way ANOVA and nonparametric Bonferroni test).

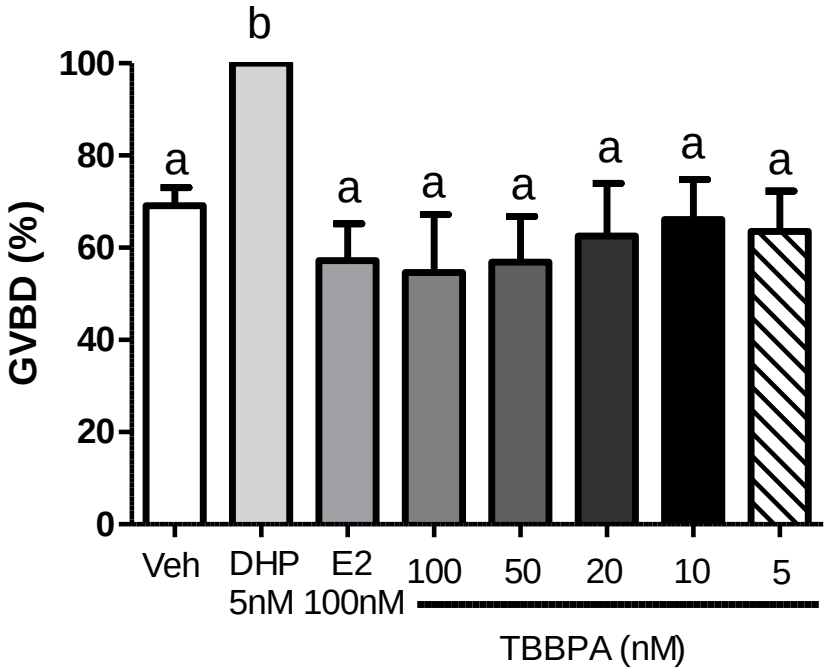


Fig. 11 Effects of various concentrations of tetrabromobisphenol A on spontaneous maturation of denuded zebrafish oocytes in the in vitro GVBD bioassay. Observation made at 5 hours. Vehicle-control ethanol; DHP- 17,20β-dihydroxy-4-pregen-3-one; E2- Estradiol-17β; TBBPA- tetrabromobisphenol A. Different letters denote significant differences from each other (p <0.05, one-way ANOVA and nonparametric Bonferroni test).

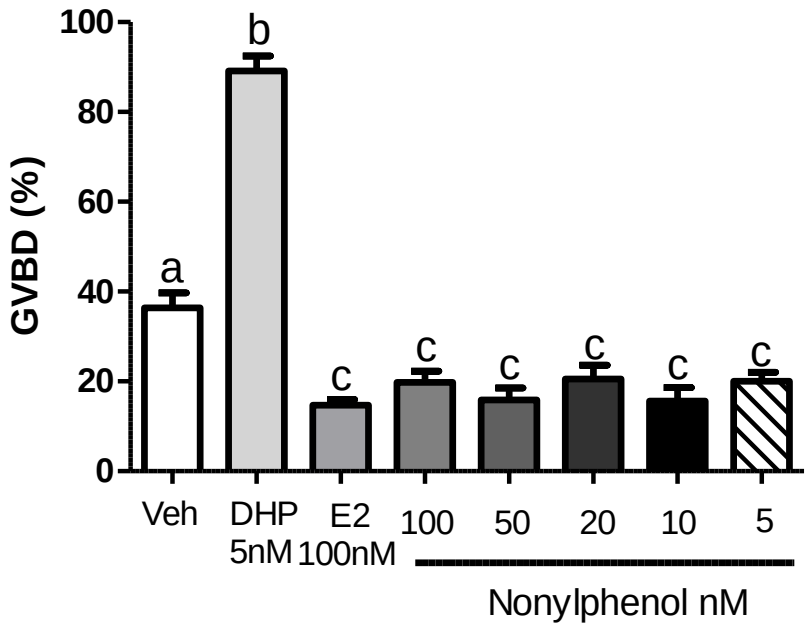


Fig. 12 Effects of various concentrations of nonylphenol on spontaneous maturation of denuded zebrafish oocytes in the *in vitro* GVBD bioassay. Vehicle-control ethanol; DHP-17,20β-dihydroxy-4-pregen-3-one; E2- Estradiol-17β. Bars denote means ±SEM. Different letters denote significant differences from each other (p <0.05, one-way ANOVA and nonparametric Bonferroni test).

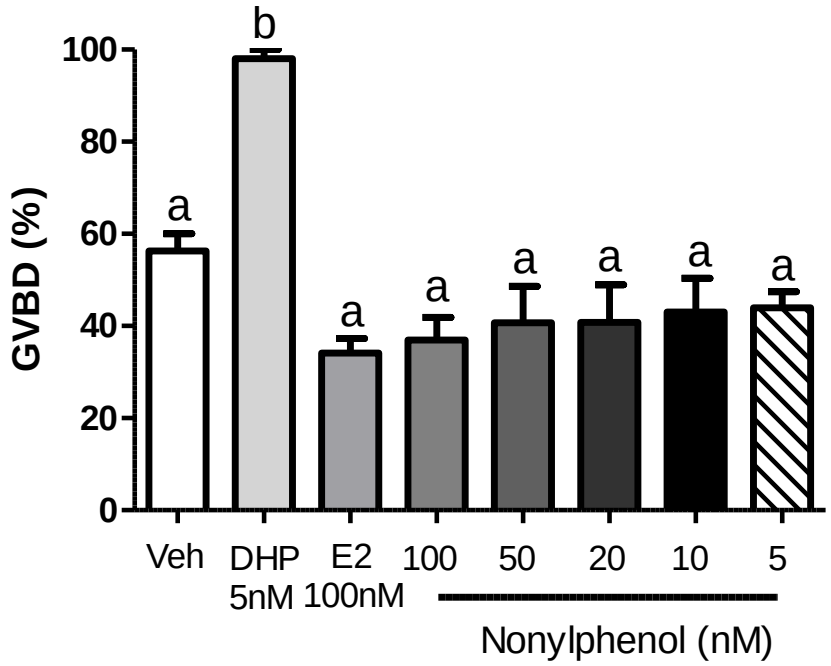


Fig. 13 Effects of various concentrations of nonylphenol on spontaneous maturation of denuded zebrafish oocytes in the *in vitro* GVBD bioassay. Observation made at 5 hours. Vehicle control ethanol; DHP- 17,20β-dihydroxy-4-pregen-3-one; E2- Estradiol-17β;. Different letters denote significant differences from each other (p <0.05, one-way ANOVA and nonparametric Bonferroni test).

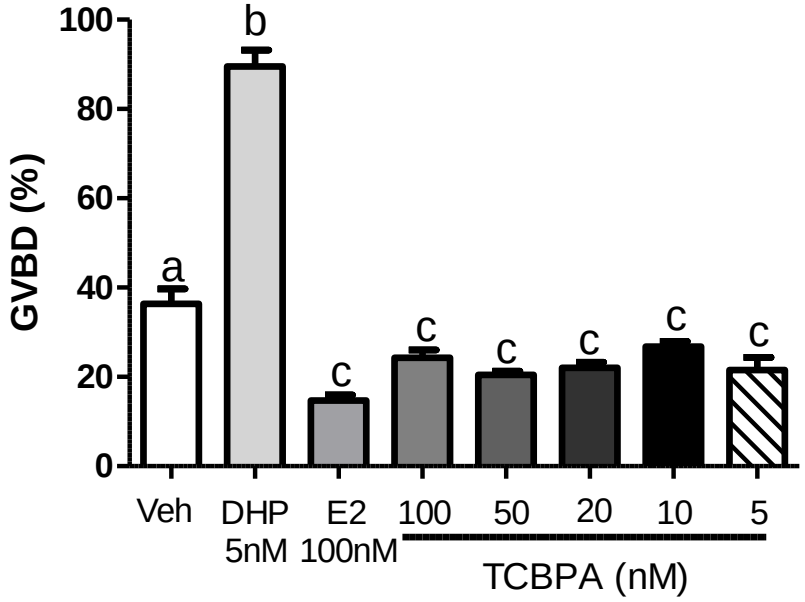


Fig. 14 Effects of various concentrations of tetrachlorobisphenol A on spontaneous maturation of denuded zebrafish oocytes in the *in vitro* GVBD bioassay. Vehicle-control ethanol; DHP- 17,20β-dihydroxy-4-pregen-3-one; E2- Estradiol-17β; TCBPA- tetrachlorobisphenol A. Bars denote means ±SEM. Different letters denote significant differences from each other (p <0.05, one-way ANOVA and nonparametric Bonferroni test).

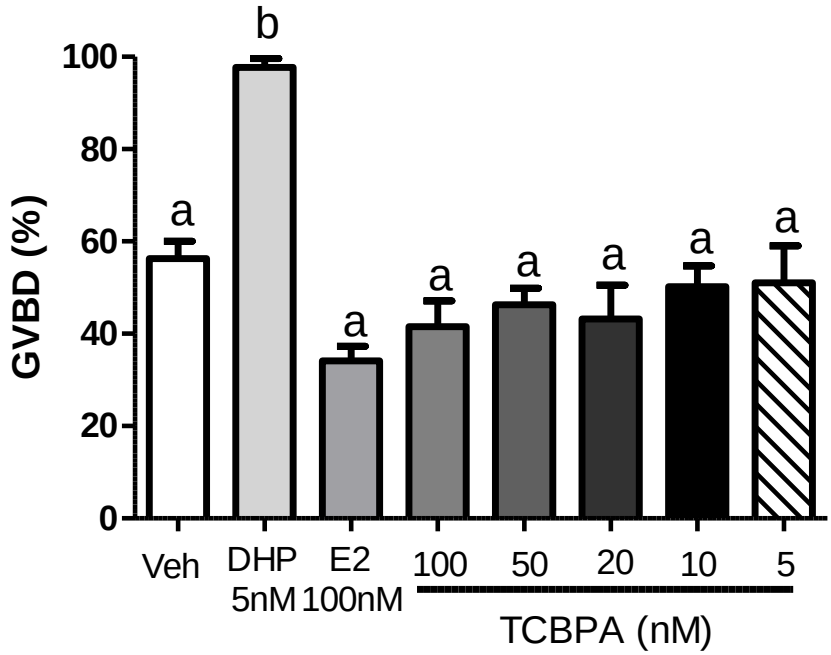


Fig. 15 Effects of various concentrations of tetrachlorobisphenol A on spontaneous maturation of denuded zebrafish oocytes in the *in vitro* GVBD bioassay. Observation made at 5 hours. Vehicle-control ethanol; DHP- 17,20β-dihydroxy-4-pregen-3-one; E2- Estradiol-17β; TCBPA- tetrachlorobisphenol A. Different letters denote significant differences from each other (p <0.05, one-way ANOVA and nonparametric Bonferroni test).

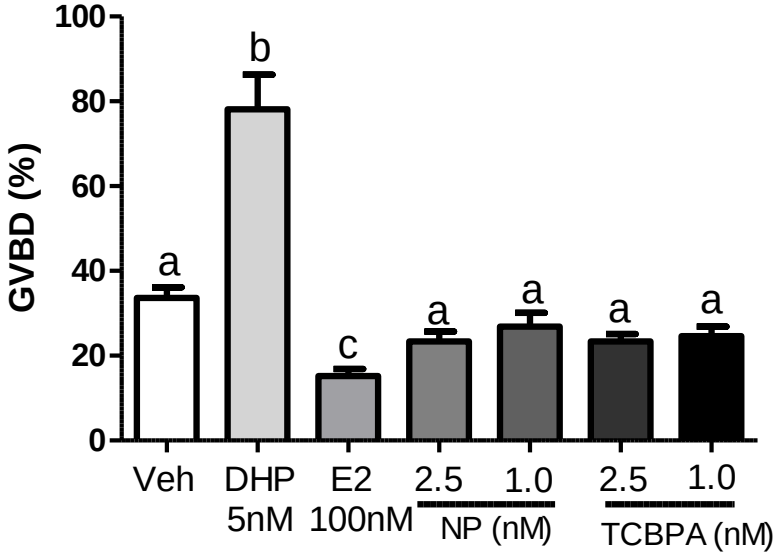


Fig. 16 Effects of low concentrations of tetrachlorobisphenol A and nonylphenol on spontaneous maturation of denuded zebrafish oocytes in the *in vitro* GVBD bioassay. Vehicle-control ethanol; DHP- 17,20β-dihydroxy-4-pregen-3-one; E2- Estradiol-17β; NP-nonylphenol; TCBPA- tetrachlorobisphenol A. Bars denote means ±SEM. Different letters denote significant differences from each other (p <0.05, one-way ANOVA and nonparametric Bonferroni test).

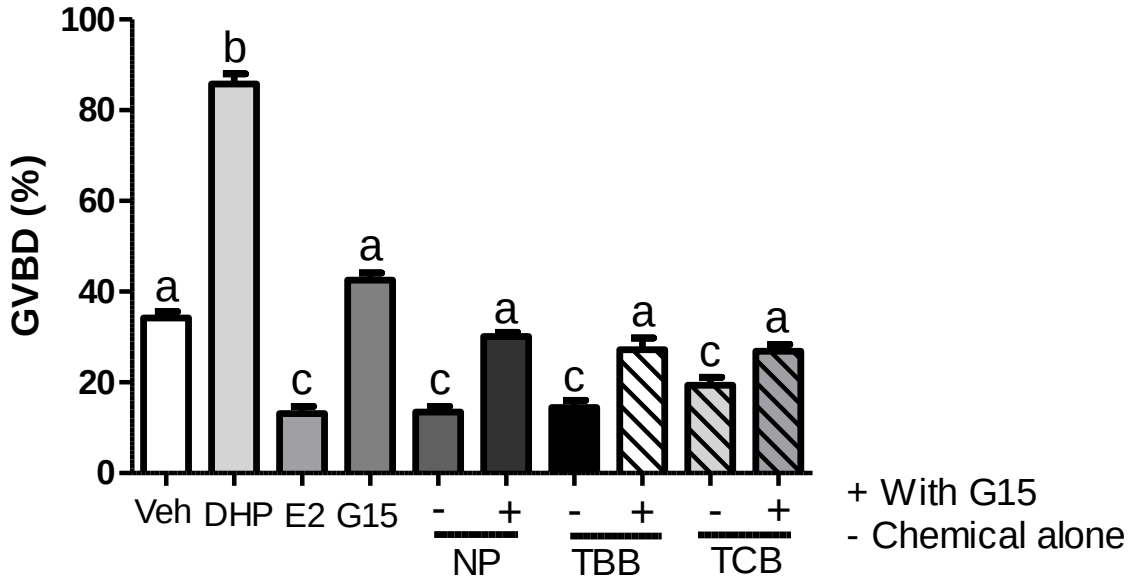


Fig. 17 Effects of the specific GPER antagonist G-15 on the inhibitory effects of alkylphenols on spontaneous maturation of denuded zebrafish oocytes in the *in vitro* GVBD bioassay. Control- vehicle control ethanol; DHP- 5nM17,20β-dihydroxy-4-pregen-3-one; E2- Estradiol-17β; G15- the specific GPER antagonist 100nM; NP- 100nM nonylphenol; NP+G- 100nM nonylphenol with 100nM G-15; TBB- 100nM tetrabromobisphenol A; TBB+G- 100nM tetrabromobispehnol A with 100nM G15; TCB- 100nM tetrachlorobisphenol A; TCB+G- 100nM tetrachlorobisphenol A with 100nM G-15. Bars denote means ±SEM. Different letters denote significant differences from each other (p <0.05, one-way ANOVA and nonparametric Bonferroni test).

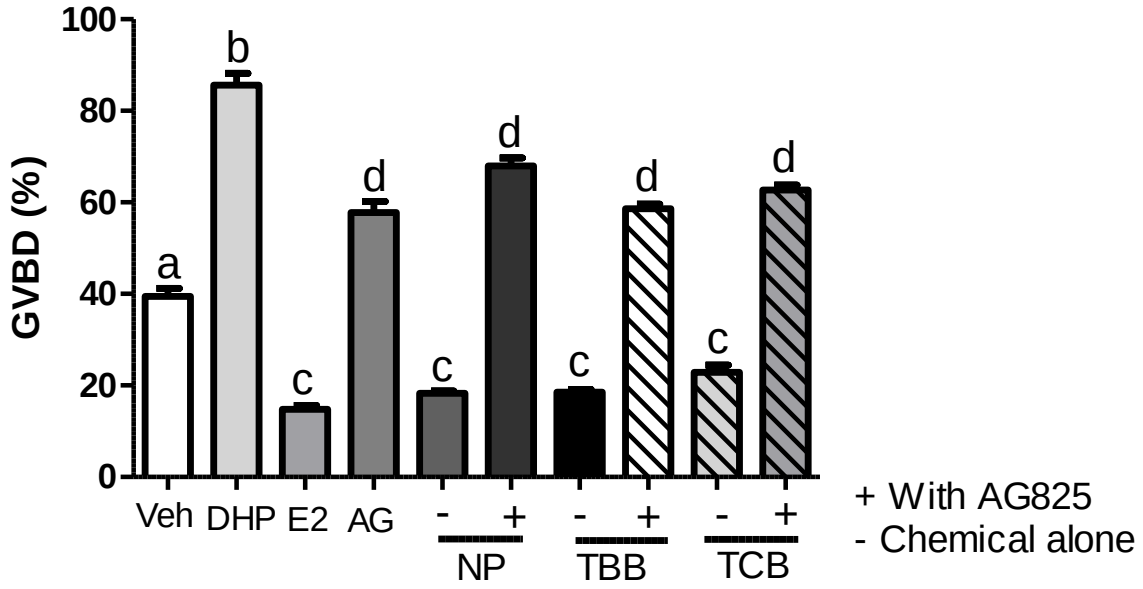


Fig. 18 Effects of the Egfr inhibitor AG825 on the inhibitory effects of alkylphenols on spontaneous maturation of denuded zebrafish oocytes in the *in vitro* GVBD bioassay. Vehicle-control ethanol; DHP- 5nM 17,20β-dihydroxy-4-pregen-3-; E2- 100nM Estradiol-17β; AG825- 50uM of the EGFR inhibitor; NP- 100nM nonylphenol; NP+A 100nM nonylphenol with 100nM AG825; TBB- 100nM tetrabromobisphenol A; TBB+A 100nM Tetrabromobispehnol A with 100nM AG825; TCB- 100nM tetrachlorobisphenol A; TCB+A- 100nM tetrachlorobisphenol A with 100nM AG825. Bars denote means ±SEM. Different letters denote significant differences from each other (p <0.05, one-way ANOVA and nonparametric Bonferroni test).

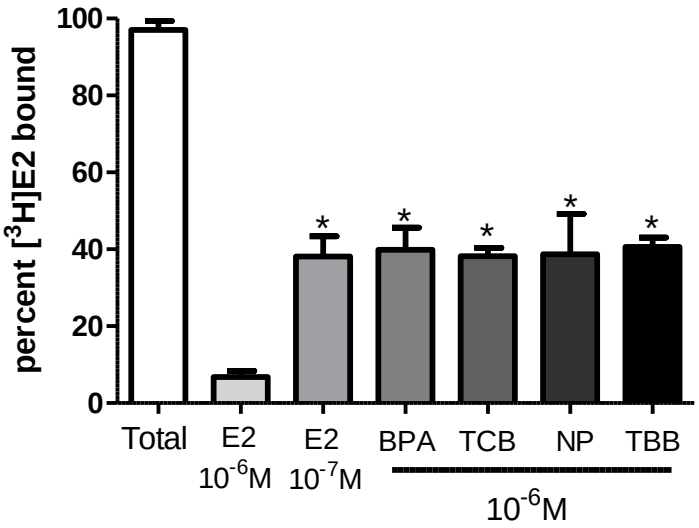


Fig. 19 Binding of alkylphenol compounds to recombinant zebrafish Gper on transfected HEK293 cell membranes in a single-point competitive binding assay. Displacement of bound [^{3}H]-E2 by 1μM BPA, TCBPA, Nonylphneol, and TBBPA compared to that displaced by 100nM and 1μM E2. Values are represented as a percent of the total binding. Total- membranes incubated with 4nM [^{3}H]-E2 alone. Bars denote mean with SEM, (*) represents significant differences from total (p <0.05, one-way ANOVA and Dunnett's test). Nonspecific binding is represented as the amount of [^{3}H]-E2 bound to receptor when 1000x more competitor than isotope is added, as seen in the E2-10^{-6}M treatment.

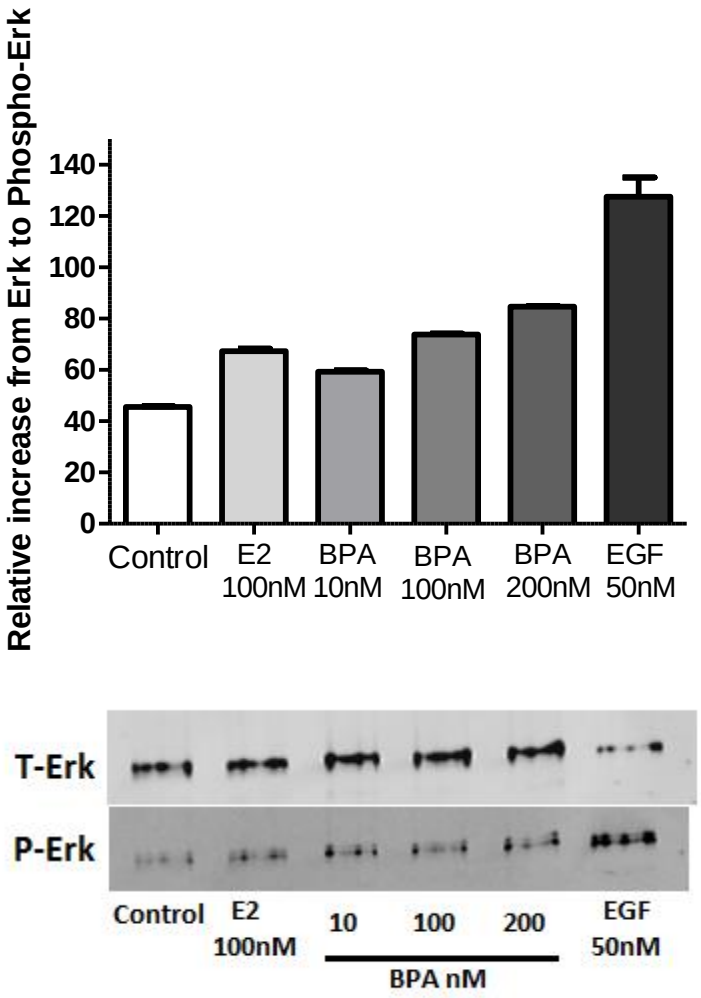


Fig. 20 Effects of Bisphenol A on Erk 1/2 phosphorylation. A. Control- vehicle control ethanol; B. E2- Estradiol-17β 100nM; BPA-Bisphenol A 10nM, 100nM, 200nM; EGF-Epidermal Growth Factor 50nM. Bars denote mean with SEM. Percent increase in phosphorylated Erk levels compared to total Erk levels determined by densitometric analysis of bands detected on the Western blots. (Phospho-Erk/t-Erk)*100= Fold increase compared to loading control, total Erk. This graph represents one experimental replicate.

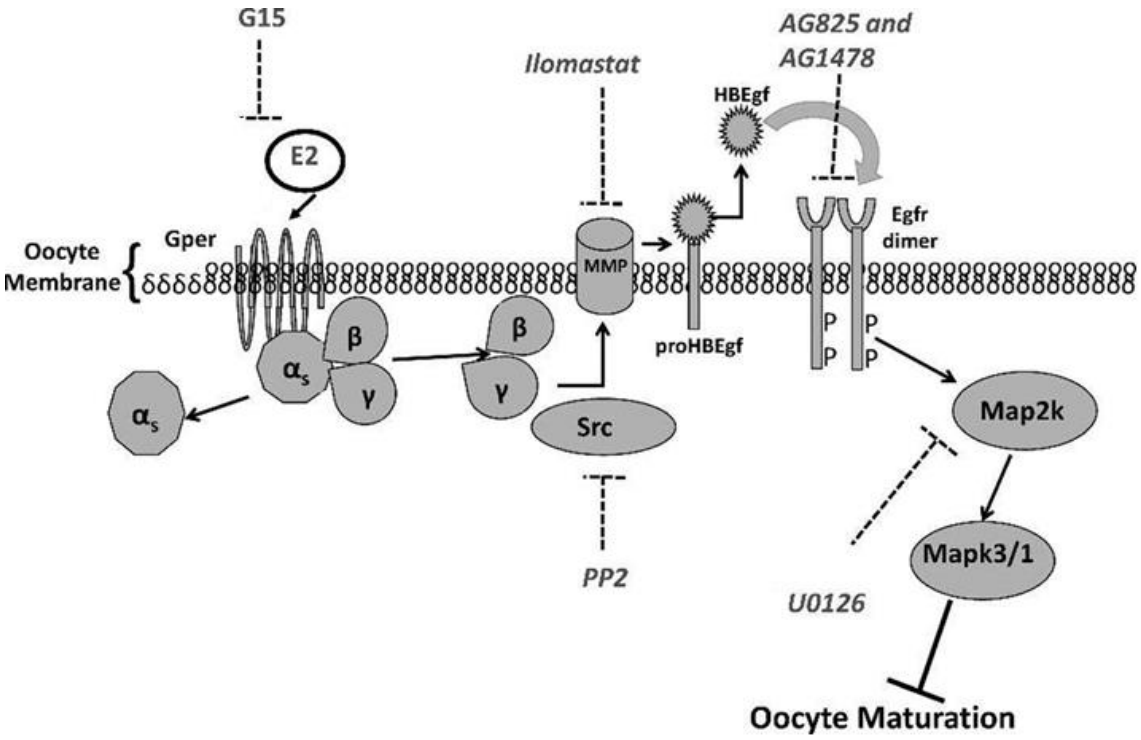
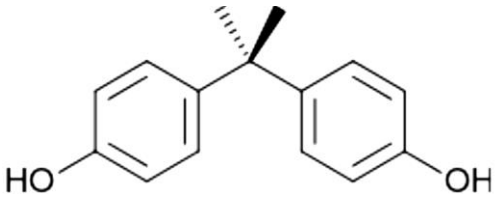
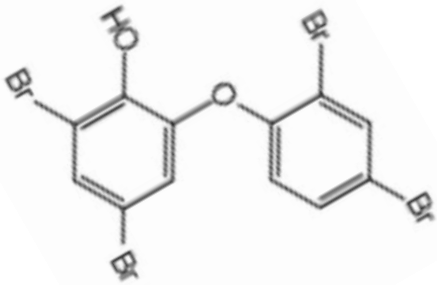


Fig. 21 Proposed mechanism of Egfr activation, adapted from Peyton and Thomas 2011.



1



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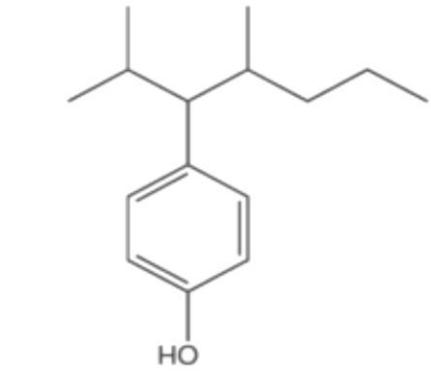

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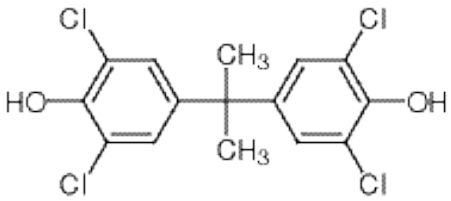

4

Fig. 22 Structure of 1. Bisphenol A, 2. TetrabromobisphenolA, 3. Nonylphenol, 4. Tetrachlorobisphenol A.

APPENDIX

G-protein coupled estrogen receptor zebrafish: Gper

G-protein coupled estrogen receptor mammal: GPER

Mitogen activated protein kinase: Mapk3/1 or Erk 1/2

Oocyte maturation: OM

Germinal vesicle breakdown: GVBD

Epidermal growth factor receptor zebrafish: Egfr

Epidermal growth factor receptor mammal: EGFR

Epidermal growth factor: EGF

Bisphenol A: BPA

Tetrachlorobisphenol A: TCBPA

Tetrabromobisphenol A: TBBPA

Nonylphenol: NP

Luteinizing hormone: LH

Estradiol-17β: E2

17,20β-dihydroxy-4-pregnen-one: DHP

Maturation inducing hormone: MIH

Membrane progesterone receptor alpha: mPRα

Cyclic adenosine monophosphate: cAMP

Nuclear estrogen receptors ERα and ERβ: nERs

Leibovitz L-15 culture media: L-15

Relative binding affinity: RBA

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