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Shortened version of title: Characterising combined effects of anti-estrogens *in vitro*

Characterising combined effects of anti-estrogenic chemicals on vitellogenin production in rainbow trout (*Oncorhynchus mykiss*) hepatocytes

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Abstract

Fish are exposed to a complex mixture of endocrine disrupting compounds, whereof some display anti-estrogenic activity leading to suppression of estrogen receptor (ER) mediated reproductive processes. Although the main Mode of action (MoA) of these anti-estrogens are direct interference with natural ligand binding of the ER, several other MoAs have been proposed. The aim of the present study was to characterise the single and combined anti-estrogenic effects of the aryl hydrocarbon receptor (AhR)-agonist β-naphtoflavone (BNF) and ER-antagonist 4-hydroxytamoxifen (OHT) on vitellogenin (Vtg) protein using primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes. Supporting transcriptional analysis of ER-responsive genes (*estrogen receptor-α* (*er-α*)*, vitellogenin-1* (*vtg-1*), eggshell *zona radiata protein* (*zrp*)) and AhR-mediated genes (*aryl hydrocarbon receptor-2β, cytochrome p450-1a* (*cyp1a*)) was

performed by qPCR to characterise the anti-estrogenic effect on ER- and AhR-mediated responses. The results displayed that both BNF and OHT reduced the 17β-estradiol (E2)-induced Vtg protein expression in a concentration responsive manner, whereas exposure to a mixture of these caused an additive anti-estrogenic effect. The results observed at the protein level was further supported by transcriptional analysis of ER-responsive genes (*er-α, vtg-1, zrp*), where only E2-induced *vtg-1* gene expression was significantly reduced by OHT and the mixture of OHT and BNF. Although not statistically significant, E2-induced *er-α* and *zrp* gene expression displayed a tendency of being reduced by OHT, BNF and the mixture of these. The significant reduction of E2-induced *vtg-1* gene expression by OHT indicated that the anti-estrogenic effect of this compound was mediated by the ER signalling pathway. Specific genes involved in putative AhR-ER cross-talk was also investigated, however none could be directly associated with the compounds anti-estrogenic MoA. Although the MoAs of the single compounds and the mixture were not completely characterised, the present study has enhanced our knowledge of the combined toxicity mediated by anti-estrogens acting by different MoA.

Key Words: Primary hepatocytes, Anti-estrogens, AhR-ER cross-talk, Vitellogenin

1 Background

Aquatic organisms are constantly exposed to mixtures of organic chemicals from various anthropogenic sources of emission (e.g. industry emission, air deposition, sewage treatment plants, land run-off etc.). These compounds affect the aquatic organisms through different modes of action (MoA), whereof some may modulate the endocrine system beyond homeostasis and cause endocrine disruption (Sumpter, 2005). Endocrine disrupting chemicals (EDCs) have

the potential to affect reproduction, immune responses, and development, raising concern for wildlife and humans (Casanova-Nakayama et al., 2011; Kavlock and Ankley, 1996; Tyler et al., 1988).

EDCs may modulate molecular targets in the endocrine system by disturbing the homeostasis of hormone-regulating processes such as the hypothalamus-pituitary-gonad (HPG) axis through the estrogen, androgen or thyroid (EAT) signalling pathways (Munn and Goumenou, 2013). The best characterised EDCs are estrogen receptor (ER) agonists, which in many tissues bind and activate the ER to initiate a series of molecular and cellular events. Upon ligand binding, the ER-ligand complex is translocated into the nucleus where it initiates transcriptional activation of the ER and modulate downstream target genes containing the estrogen responsive element (ERE) (Filby and Tyler, 2005; Matthews and Gustafsson, 2003; Shanle and Xu, 2011). In female fish, activation of the ER induces vitellogenesis in the liver by increasing the transcriptional activity and synthesis of the egg-yolk precursor protein vitellogenin (Vtg), eggshell zona radiata protein (Zrp), vigilin and follistatin (Arukwe et al., 2000; Hyllner et al., 1991). The synthesized proteins (e.g. Vtg and Zrp) are then transported via the blood stream to the gonads where they have an essential role in vitellogenesis and oogenesis (Tyler et al., 1988). Vitellogenesis do not normally occur in juvenile or male fish, consequently these genes and proteins are used as estrogenic biomarkers in liver or blood to detect chemicals that interfere with ER signalling pathways (Sumpter and Jobling, 1995; Tollefsen et al., 2003).

Although, the main research has been on ER-agonistic compounds, several organic pollutants exert anti-estrogenic effects by suppressing the expression of the ERs, Vtg, Zrp and vigilin in fish (Gräns et al., 2010; Mortensen and Arukwe, 2008; Petersen and Tollefsen, 2012; Rankouhi et al., 2004). Some of these anti-estrogenic compounds cause adverse effects such as masculinization and reduced fecundity in female fish (Andersson et al., 1988; Bugel et al., 2011; King Heiden et al., 2006). Directly acting anti-estrogens, such as estrogen receptor antagonists

e.g. 4-hydroxytamoxifen (OHT) and fluvestrant (ICI 182,780), bind to the ER, disrupt the ER signalling in target tissues and interfere with normal activity of co-regulators (Dobrzycka et al., 2003). The ER-antagonists may also bind to the activation function 1 (AF1) and/or 2 (AF2) in the ligand binding domain (LBD) of the ERE, causing full (AF1 and AF2) or partial (AF2) inhibition of ER transcription (for full review see Aranda and Pascual, 2001). Interestingly, all chemicals with anti-estrogenic properties are not necessarily causing their effects through a ligand-binding mechanism, but may elicit anti-estrogenicity by a receptor cross-talk (Matthews and Gustafsson, 2006). One type of cross-talk has been described in several *in vitro* and *in vivo* models (for full review see Matthews and Gustafsson, 2006; Safe and Wormke, 2003; Swedenborg and Pongratz, 2010) as a uni- or bi-directional cross-talk between the aryl hydrocarbon receptor (AhR) and ER (Gräns et al., 2010; Mortensen and Arukwe, 2007). This AhR-ER cross-talk involve several mechanisms including AhR-mediated metabolism of estrogens (Safe and Wormke, 2003), AhR-mediated competition for common transcriptional co-regulators (Brunnberg et al., 2003; Rüegg et al., 2008), and AhR-mediated proteasome degradation of the ER (Bemanian et al., 2004; Ohtake et al., 2003; Safe and Wormke, 2003). Although the general mechanisms of the AhR-ER cross-talk has been proposed in mammals (Safe and Wormke, 2003) and fish (Bemanian et al., 2004; Gräns et al., 2010; Mortensen and Arukwe, 2007; Navas and Segner, 2000), detailed knowledge of how different anti-estrogens cause effects when present in complex mixtures are still unclear. It is becoming increasingly clear that the combined effects of anti-estrogenic compounds are complex (Mortensen and Arukwe, 2007), and demonstration of synergy between classical pollutants and ER-antagonists on the suppression of ER-mediated Vtg production in fish bioassays (Petersen and Tollefsen, 2012) suggest that effort to characterise the MoA of these anti-estrogens and mixtures of these are highly warranted.

The aim of the study was to characterise the anti-estrogens β-naphtoflavone (BNF) and 4-hydroxytamoxifen (OHT) modulatory effects on ER-responsive genes (*estrogen receptor-α* (*er-α*), eggshell *zona radiata protein* (*zrp*), *vitellogenin -1* (*vtg-1*)) and Vitellogenin protein (Vtg) in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes, when exposed separately and in combination. Genes associated with the AhR-activity (*aryl hydrocarbon receptor 2β* (*ahr2β*), *cytochrome p450 1a* (*cyp1a*)) were also monitored to determine potential AhR-ER cross-talk. Transcriptional and protein analysis, using quantitative (real-time) polymerase chain reaction (qPCR) and enzyme-linked immunosorbent assay (ELISA) were used to characterise the anti-estrogenicity of single chemicals and a mixture of these.

2 Material and methods

2.1 Chemicals and exposure concentrations

The chemicals, β-naphtoflavone (BNF >95%, CAS: 6051-87-2), 4-hydroxytamoxifen (OHT ≥98%, CAS: 68047-06-3), 2,3,7,8-tetrachlorodibenzo-pdioxin (TCDD, CAS: 1746-01-6) and 17β-estradiol (E2, CAS: 50-28-2) were obtained from Sigma Aldrich (St Lois, MI, US). All compounds were dissolved in DMSO and kept at -20°C when not in use.

2.2 Fish

Rainbow trout (200-500g) were obtained from the Valdres rakfisk AB hatchery (Valdres, Norway) and reared at the Department of Biology, University of Oslo (Norway) for a minimum of 4 weeks prior to the study. The donor fish used in the study were from the same fish stock, maintained in aged tap water from lake Maridalsvannet (Oslo, Norway) at 6±2°C, pH 6.6, 100%

oxygen saturation and light regime of 12h light/12h dark. Rainbow trout were fed daily with 50/50 mix of Protec and Spirit commercial pellets (Skretting, Stavanger, Norway) corresponding to approximately 0.5% of total body weight.

2.3 Primary cultures of rainbow trout hepatocytes, exposure, and sampling

A total of 7 juvenile/male rainbow trout, size 200-500 grams, were terminated by a blow to the head followed by visual inspection of the maturity status of the gonads. Only juvenile and male fish with no visual development of the gonads were subjected to liver perfusion. The livers of the 7 fish were perfused using a two-step perfusion method, described in Tollefsen et al. (2003). The viability of the cells was assessed by the trypan blue exclusion test, and only cell isolations with ≥ 90% viability were used for the experiments. Cells were diluted to a final concentration of 500 000 cells/ml and seeded in 24-well plates (1.25 ml/well). After 24h of acclimatisation, cells were exposed to the test compounds (singly and in combination) and DMSO control. Cells were re-exposed after 48h to maintain exposure concentrations as shown to be crucial to obtain high-quality concentration-response curves (CRC). Cells and cell culture media were sampled at 48h and 96h for gene and protein analysis, respectively. At the end of exposure, cell culture media was transferred in triplicates (3 x 100µl) to a Maxisorb nunc-immunoplate (Nunc, Roskilde, Denmark), and frozen at -80°C for subsequent vitellogenin analysis, whereas cells were sampled for subsequent RNA isolation according to the supplier instructions from the Qiagen RNeasy Plus mini kit (Qiagen GmbH, Hilden, Germany).

2.4 Experimental design

In order to characterise the MoA of BNF and OHT, two different exposure designs were used (table 1). The compounds BNF (AhR-agonist) and OHT (ER-antagonist) were chosen on basis of their differences in MoA and potential interference with E2-induced Vtg protein expression. Testing for the 50% ER-agonistic inhibitory concentration (IC50) was performed in experiment 1 to obtain the parameters necessary to design a mixture of BNF and OHT (given in combination with a fixed concentration of E2 of $6.3E^{-10}$ mol/L). This would make it possible to assess whether the inhibitory effects on E2-induced Vtg protein expression observed after exposure to the mixture of BNF, OHT and E2 could be explained by additivity. Cells from three individual cell isolations were exposed to a dilution series of BNF and OHT in presence of a sub-lethal E2 concentration (E2: $6.3E^{-10}$ mol/L) causing 50% induction of Vtg protein production.

Experiment 2 was performed after the preliminary identification of IC_{50} values for BNF and OHT in the 24 well format, using four individual cell isolations and exposing them to individual compounds and binary mixtures of the anti-estrogenic compounds in absence (E2, BNF, OHT, BNF + OHT) and presence of E2 (E2 + OHT, E2 + BNF, E2 + OHT + BNF), in addition to the solvent control (DMSO 0.1%).

2.5 Enzyme linked immunosorbent assay (ELISA)

Vitellogenin was measured in the primary hepatocyte media by a capture (semi-quantitative) ELISA, previously described by Tollefsen et al. (2003). In brief, the frozen microtiter plates containing media sampled after 96h of exposure, were thawed at 4°C, followed by application of 100 µl of positive standard dilution (rainbow trout Vtg) in empty wells and further incubated in the dark over night at 4°C (>16h). The capture ELISA was performed using a monoclonal

mouse anti-salmon Vtg (BN-5, Biosense Laboratories, Bergen, Norway) antibody, followed by the secondary antibody goat anti-mouse IgG (Bio-Rad, Hercules, CA, USA), both applied in a 1:6000 dilution and incubated 2h and 1h, respectively. A HRP enzyme substrate (TMB plus, KEMENTEC diagnostics, Taastrup, Denmark) was added to the wells following appropriate washing to start the colour development. After 15 minutes of incubation in the dark at room temperature, the reaction was stopped by adding 50 µl of 1M H_2SO_4. The absorbance was measured within 20 min at 450 nm using a Thermomax microplate reader (Molecular Devices, USA). The relative Vtg expression was calculated as percentage of maximum Vtg induction ($6.3E^{-10}$M E2) at 96 h of exposure by normalising the individual treatment against the basal expression in the DMSO control.

2.6 Quantitative Real time PCR (qPCR)

The gene expression analysis was performed using quantitative (real-time) polymerase chain reaction (qPCR). Synthesis of cDNA was performed through reverse transcription of total RNA (0.5-1 µg) using Quanta qScript™ cDNA Synthesis Kit (Quanta Biosciences Inc., Gaithersburg, USA) according to the manufacturer's instructions and outlined for use with primary rainbow trout hepatocytes in Hultman et al. (2015a). The primer optimisation was performed using a template pool consisting of a 5-step dilution series (5-100 ng/reaction) in a 384 well plate format on a CFX-384 thermal cycler (Bio-Rad laboratories Inc., USA). SYBR®Green Supermix fluorescence dye (Quanta Biosciences Inc., Gaithersburg, USA) was used in the qPCR amplification reaction, where duplicates of 10 ng template/well was used in the final mastermix reaction (20 µl/reaction). The primers were designed using the NCBI accession number corresponding to the housekeeping and target genes (Table 2) and the primer design software "Primer 3 Input version 0.4.0" and were produced by Eurofins MWG synthesis GmbH (Ebersberg, Germany). To exclude suspicions of contamination of the mastermix or presence

of genomic DNA in the sample all tested genes had a non-template control (NTC) and a no-reverse transcriptase control (NRT). Primer dimers or unspecific amplified products were also assessed by applying a melting curve at the end of the qPCR analysis. Accepted quantification cycle (Cq)-value of NTC was set to be either non-detectable (N/A) or Cq-value >30 with minimum 7 cycles in between template replicate and NTC. Ubiquitin was stable in all treatments and was therefore used as a housekeeping gene (table 2). Data normalisation was performed using the Pfaffl method (Pfaffl, 2001).

2.7 Data analysis

2.7.1 ELISA and qPCR analysis

The Vtg protein data after exposure to BNF or OHT in combination with a fixed concentration of E2 were normalised between the response of E2 alone ($3E^{-8}$ mol/L, 100 %) and the DMSO control (0%). Data from the dilution series of BNF and OHT was modelled with a sigmoidal dose-response curve with variable slope in Graphpad Prism v5.04 (Graphpad Software, Inc., San Diego, CA, USA).

All qPCR data were normalised against the reference gene ubiquitin and stated as relative expression prior to statistical analysis. The gene expression data for ER-signalling was normalised between the cell batch-specific response of $1E^{-8}$ mol/L E2 alone (100 %) and the DMSO control (0%), whereas data for AhR-signalling was normalised against their individual cell media control and presented as fold change due to low response to the positive control $3E^{-10}$ mol/L TCDD. The statistical analysis was performed using Graphpad Prism. Prior to any statistical tests, all data were log-transformed to meet the criteria's of normality, and a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test were used to identify significant differences in the treatments ($p < 0.05$). To investigate whether there was an

interaction amongst the tested treatments a two-way ANOVA was performed followed by Bonferroni post hoc test, with a significant p-value set at <0.05.

2.7.2 Evaluation of mixture effects on Vtg protein expression

Evaluation of combined effects was performed on the Vtg protein expression after 96h exposure. The concentration addition (CA, Eq. 1) and independent action (IA, Eq. 2) prediction models were used to assess the inhibitory mixture effect of BNF and OHT on the E2-induced Vtg protein expression. The initial concentration response curve parameters from experiment 1 were used to design an equitoxic mixture of OHT and BNF based on the IC50 ratios. The final CRC parameters for the single compounds from experiment 2 was used in the prediction models to calculate the predicted combined effect of the applied mixture compositions.

$$ECx_{mix} = \left(\sum_{i=1}^{n}\left(\frac{p_i}{ECx_i}\right)\right)^{-1} \qquad \text{Eq. 1}$$

$$E_{mix} = 1 - \prod_{i=1}^{n}(1 - E_i) \qquad \text{Eq. 2}$$

ECx_{mix}: the predicted total concentration of the mixture that produces x% effect

p_i: the relative fraction of component i in the mixture

ECx_i: is the concentration of substance i provoking the effect x when applied alone.

E_{mix}: the effect of the mixture

E_i: the effect of substance i when applied alone.

Additivity was assumed if the predicted effects where within the 95% confidence interval (CI) of the experimental data and/or if the model deviation ratios (MDRs, the ratio between the predicted effect concentration and the experimental effect concentration at a certain effect level) was within a factor of two ($0.5 \leq MDR \leq 2$).

3 Results

Expression of Vtg protein levels and a selection of relevant genes was analysed to characterise the anti-estrogenic effects of the compounds individually and in a mixture. All relevant data are presented as figures herein, and further details may be found as supplementary information (Supplementary Figure 1, 2, 3 and Table 1 and 2).

3.1 Effects on ER signalling

3.1.1 Vitellogenin protein inhibition single compounds

The two differently acting anti-estrogenic compounds reduced E2-induced Vtg protein expression in a concentration dependent manner (Fig. 1) with 50% inhibition concentrations (IC50) of $1.05E^{-7}$ M (BNF) and $7.60E^{-9}$M (OHT). The potency of BNF and OHT differed by a factor of 14 with OHT being the most potent anti-estrogen. The cell viability measured as cell membrane integrity (data not shown) and metabolic activity (Fig. 1) was only significantly affected at $1.0E^{-7}$M (88% metabolic activity) for OHT. Although the highest concentration of OHT reduced the metabolic activity to 81%, this concentration was approximately 1000 times higher than the IC50 value and was not considered to affect the sub-lethal responses.

3.1.1.1 Vitellogenin protein inhibition of mixture

17β-estradiol ($6.3E^{-10}$M) caused a significant induction of Vtg protein expression (44% of positive control of 30 nM E2) compared to the DMSO control after 96h of exposure (Fig. 2). The E2-induced Vtg protein expression was significantly reduced to 29% and 23% of the positive control by BNF ($2.38E^{-8}$ M) and OHT ($1.1E^{-8}$ M), respectively. The binary mixture of these two (BNF+OHT) significantly reduced protein expression to 10% of positive control, and verified that all the anti-estrogenic treatments suppressed ER-mediated Vtg protein expression. The two-way ANOVA identified treatments containing BNF to cause a significant reduction ($P = 0.0022$) of E2-induced Vtg protein (Fig. 2), albeit no significant effect of OHT (one-way ANOVA) or interaction was identified between the compounds BNF and OHT by two-way ANOVA analyses (Supplementary table 2). Due to the compounds' suppressive effect on vitellogenesis, further investigation of potential anti-estrogenic MoAs and potential interactions was performed on a suite of ER-mediated gene responses.

3.1.1.2 Combined effects assessment

To assess whether the observed effects of the combination of OHT and BNF were due to additivity or any type of interaction between BNF and OHT, the data were normalised between the DMSO control (0%) and the E2 concentrations ($6.3E^{-10}$ mol/L) used when developing CRCs for BNF and OHT. The predicted effects of the mixtures were calculated by CA and IA which differed by a factor of 1.4 with IA predicting stronger anti-estrogenic effect than CA (Fig. 3, Suppl. Table 1). The calculated MDRs (all within a factor of 2) indicated that the mixture of OHT and BNF had an additive effect on the inhibition of E2-induced Vtg protein expression at the tested concentrations and mixture ratio. It should be noted that Vtg data are based on a semi-quantitative ELISA analysis, which does not reflect actual levels of Vtg in the medium, but depict the change relative to maximum and minimum induction levels.

3.1.2 Estrogen receptor-α transcription

17β-estradiol ($6.3E^{-10}$ mol/L) caused a significant hepatic induction of *er-α* gene expression (59%) compared to the DMSO control after 48h exposure (Fig. 4). Although not statistically significant, a tendency to reduce the E2-induced *er-α* gene expression after exposure to BNF (11% reduction), OHT (21% reduction) and BNF+OHT (33% reduction) was observed. As seen for the Vtg protein expression, a tendency for reduced *er-α* gene expression occurred in all treatments indicative of an anti-estrogenic MoA. The two-way ANOVA identified that OHT was significantly different ($p=0.0332$) from E2 (Fig. 4), albeit no significant anti-estrogenic effect was identified for BNF or the interactions among the compounds (Suppl. Table 2). In absence of E2, *er-α* gene expression was unaffected by BNF, OHT, and the mixture of these (BNF+OHT) (Suppl. Fig. 1).

3.1.3 Vitellogenin-1 transcription

17β-estradiol ($6.3E^{-10}$ mol/L) caused a significant induction of *vtg-1* gene expression (15%) compared to the DMSO control (Fig. 5). A significant reduction of the E2-induced *vtg-1* gene expression was observed after exposure to OHT (11% reduction) and OHT + BNF (13% reduction), whereas a non-significant tendency for reduction (6%) was observed after exposure to BNF. The two-way ANOVA revealed that treatments containing BNF caused a significant ($p=0.0032$) reduction in E2-induced *vtg-1* expression (Fig. 5, Suppl. Table 2). No interaction was observed between the two compounds (BNF and OHT) when assessed as *vtg-1* expression (Suppl. Table 2). In the absence of E2, no statistical difference between the DMSO control and BNF, OHT, and a mixture of these were observed on the *vtg-1* gene expression (Suppl. Fig. 1).

3.1.4 Eggshell zona radiata protein transcription

The ER-mediated gene expression of *zrp* was significantly induced (23%) after exposure to E2 ($6.3E^{-10}$ mol/L) (Fig. 6). Although not statistically significant, BNF, OHT and the mixture of these (BNF+OHT) displayed a tendency to reduce the E2-induced *zrp* gene expression with 4%, 13% and 17%, respectively. This trend was similar to that reported for *er-α*, *vtg-1* transcription and the Vtg protein expression herein. The two-way ANOVA revealed that no treatments caused significant reduction of E2-induced *zrp* expression compared to the control (E2 alone), and no significant interactions were observed between the two compounds (Fig. 6, Suppl. Table 2). In absence of E2, the compounds BNF, OHT and a mixture of these (BNF+OHT) did not affect *zrp* expression at the concentrations tested (Suppl. Fig. 1).

3.2 Aryl hydrocarbon receptor signalling

3.2.1 Aryl hydrocarbon receptor 2 beta transcription

The typical AhR-agonist BNF caused no induction of *ahr2β* gene expression in presence of E2 compared to the DMSO control (Fig. 7). None of the compounds (OHT, BNF, E2) or the mixture (BNF+OHT) caused an effect on *ahr2β* gene expression, which cohere with results from cells co-exposed with E2. The two-way ANOVA revealed that there was a significant ($p=0.0112$) interaction between BNF and OHT in absence of E2 (Fig. 7, Suppl. Table 2), albeit no interactions were observed between the compounds on *ahr2β* gene expression in presence of E2.

3.2.2 Cytochrome P450 1a transcription

No statistical significant changes in the cyp1a gene expression was observed for any of the treatments. However, BNF and BNF+OHT displayed a (non-significant) tendency to induce *cyp1a* gene expression when cells were co-exposed with E2 (1.76 and 2.15 fold change from DMSO control, respectively) (Fig. 8). Cells exposed to BNF and BNF+OHT in the absence of E2 also showed a tendency to increase *cyp1a* gene expression (3.08 and 1.37 fold change, respectively) (Suppl. Fig. 2, Suppl. Table 2). The two-way ANOVA demonstrated that none of the treatments caused a significant induction of *cyp1a* gene expression, nor any interaction between the two, when tested in absence and presence of E2 (Fig. 8, Suppl. Fig. 2, Suppl. Table 2). The *cyp1a* transcription was unaffected by co-exposure of E2.

4 Discussion

Understanding the combined effects of ER agonists and antagonists has become increasingly important when addressing complex mixtures and environmental samples (Grung et al., 2007; Petersen and Tollefsen, 2012, 2011). Alternative (*in vitro*) test methods such as primary hepatocytes have emerged as viable alternatives to *in vivo* testing of ER-agonists and antagonists as the hepatocytes retain native liver functions such as biotransformation activity, AhR- and ER-mediated responses (Hultman et al., 2015a, b; Pedersen and Hill, 2000; Petersen and Tollefsen, 2012; Segner and Cravedi, 2000). The hepatocytes are also suitable for screening complex mixtures of ER- and AhR-agonists and antagonists (Navas and Segner, 2006, 2000, Petersen and Tollefsen, 2012, 2011). These combined toxicity studies have demonstrated that combinations of ER-agonists predominantly act in an additive manner, whereas combinations of ER-antagonists may also act synergistically when measured as changes to Vtg protein expression (Petersen and Tollefsen, 2011, 2012). Although clear

documentation for deviations from additivity has been reported for certain mixtures, mechanistic understanding for the underlying MoA is still poorly developed in fish. The present study therefore assessed the AhR-agonist BNF and ER-antagonist OHT single and combined anti-estrogenic effects on ER-mediated genes and protein and AhR-mediated genes, in an attempt to explain how the anti-estrogenic effect is mediated and if it is due to a potential cross-talk between the receptor/receptor's signalling systems.

4.1 ER signalling

The present study showed that exposure to BNF and OHT reduced E2-induced Vtg protein expression in a concentration-responsive manner, findings that are similar to previous studies with primary hepatocytes (Gräns et al., 2010; Navas and Segner, 2000; Smeets et al., 1999). The present study verified that the anti-estrogenic effect of the compounds was dependent on their ability to modulate the activity of the ER without affecting the viability, as suppression of E2-induced Vtg protein expression previously has been suggested caused by cytotoxicity (Navas and Segner, 2000).

The currently available studies display that *in vitro* Vtg protein expression in fish hepatocytes may accommodate multiple anti-estrogenic mechanisms (e.g. ER-antagonism, receptor cross-talk), but none of these studies has fully characterised the anti-estrogenic MoA (Gräns et al., 2010; Navas and Segner, 2001; Petersen and Tollefsen, 2012). Analyses of ER-mediated (*er-α, vtg-1* and *zrp*) transcriptional responses were therefore conducted in the present study to assess the role of two anti-estrogens with different MoA and how these act in combination to cause an anti-estrogenic effect.

In the present study, E2-induced hepatocytes co-exposed with BNF and OHT displayed similar tendencies of transcriptional expression of *er-α*, *vtg-1* and *zrp* as observed for the Vtg protein expression. Several of the treatments (OHT and OHT + BNF) significantly reduced both Vtg gene and protein expression in a similar manner, illustrating the close coherence between the gene and protein responses of this estrogenic biomarker (Hultman et al., 2015). The well-characterised anti-estrogen OHT competitively bind to the ER and act as a partial ER-antagonist (Macgregor and Jordan, 1998; Li et al., 2014) and agonist (Wu et al., 2005) in different *in vivo* and *in vitro* assays from mammals and in fish. The present study concluded that OHT alone did not significantly induce any of the ER-target genes (*er-α, zrp*, *vtg-1*) (Supplementary Fig. 1) or Vtg protein, findings that are consistent with OHT displaying pure ER-antagonistic properties in fish hepatocytes (Petersen and Tollefsen, 2012).

Rainbow trout hepatocytes co-exposed with BNF and E2 displayed a non-significant tendency to reduce *er-α*, *vtg-1* and *zrp* transcription and a significant reduction of Vtg protein expression. This is in agreement with documentation of BNF being an anti-estrogen in fish hepatocytes elsewhere (Gräns et al., 2010; Navas and Segner, 2000; Petersen and Tollefsen, 2012; Smeets et al., 1999). There is currently limited knowledge on the anti-estrogenic MoA of compounds such as BNF, as they are not classic ER-ligands (Arcaro et al., 1999; Ebright et al., 1986). The anti-estrogenic effect of BNF is suggested to be caused by AhR-mediated mechanisms that suppress the activity of ER (Navas and Segner, 2000), and several AhR-agonist metabolites have weak affinity for ER by competitively binding to and initiating or inhibiting transcription (Ebright et al., 1986; Tran et al., 1996). BNF and/or its potential metabolites did not induce ER-mediated activity of *er-α, zrp* and *vtg-1* nor suppressed the basal activity of these genes when exposed alone. BNF was only capable of causing anti-estrogenic effects in presence of E2, thus suggesting that the compound's modulatory properties interfere with activation of down-stream genes in the ER-signalling pathway.

The largest anti-estrogenic effect was observed after exposure to the mixture of BNF+OHT, where the mixture reduced hepatocyte Vtg protein production significantly compared to the individual BNF and OHT treatments co-exposed with E2. This was expected based on the predicted additive effect using the CA and IA models. Mixtures of differently acting anti-estrogens (ZM 189.154 and BNF, and ZM 189.154 and PCB 126) have previously caused more than additive inhibition of the E2-induced Vtg protein expression in teleost hepatocytes (Petersen and Tollefsen, 2012). No clear deviation from additivity was observed in the present study with BNF and OHT, and may suggest that one or more of the compounds tested by Petersen and Tollefsen, 2012 were activating processes that gave rise to synergy, whereas the combination of BNF and OHT tested herein did not. In addition to the significantly reduced transcriptional expression of *vtg-1*, the tendency of reduced transcriptional expression of *er-α,* and *zrp* in the hepatocytes were also considered to be consistent with the Vtg protein expression. The binary mixture's combined anti-estrogenic effect on the ER-signalling pathway was likely due to the interference with the binding to the ER (i.e. OHT) and by modulating ER-mediated signalling (i.e. BNF), potentially through interference of the AhR-signalling pathway.

4.2 AhR signalling

AhR-agonists such as BNF bind to and activate the nuclear dioxin/xenobiotic response elements (DRE/XRE), which initiates AhR-mediated transcription of *ahr* and down-stream genes such as *cyp1a*. Transcription of *cyp1a* in turn causes increased CYP1A expression that increase the metabolism of various substrates for this biotransformation enzyme, including E2 in fish (Scornaienchi et al., 2010). Although not statistically significant, cells co-exposed with BNF and E2 displayed an apparent trend of reduction in ER transcription when compared to E2 exposed cells. Confirmation of whether this effect was caused by interference with E2

metabolism was not determined herein. In previous studies, cells co-exposed with BNF and E2 induced EROD activity and *cyp1a* transcription without affecting the metabolism of E2 in primary rainbow trout hepatocytes (Navas and Segner, 2000). Conversely, no significant effect on the *cyp1a* transcription in cells exposed to BNF alone or in combination with E2 were observed, thus indicating that neither *ahr2β* nor *cyp1a* were affected by the presence of E2 at the concentrations tested (Suppl. Fig. 2).

The OHT anti-estrogenic effect in fish has previously been ascribed to its inhibitory properties on the ER itself (Petersen and Tollefsen, 2012; Mortensen and Arukwe, 2007). The present study further confirmed these findings as no transcriptional activation of *ahr2β* or *cyp1a* by OHT in presence or absence of E2 in rainbow trout hepatocytes was demonstrated at the concentrations tested. However, the two-way ANOVA identified that OHT interfered with BNF-induced *ahr2β* transcription when cells were co-exposed with E2. A mammalian *in vitro* study reported suppression of TCDD (AhR-agonist) induced EROD activity when cells were co-exposed with OHT, thus suggesting that OHT may hinder the formation of the ligand/AhR complex (Lai et al., 2004). It has also been proposed that OHT reduce AhR-mediated signalling and EROD activity through inhibition of protein kinase C (Long et al., 1998; Lai et al., 2004). As *ahr2β* was not clearly effected by the treatments, likely due to the low concentration of BNF (2.38E-8 mol/L) used, the present study could not verify whether OHT specifically modulate the transcriptional AhR activity. Additionally, the tendency of induced *cyp1a* transcription caused by the binary mixture of BNF+OHT was similar to that observed when exposed to BNF alone and in presence of E2. These results suggest that BNF alone caused the tendency for transcriptional AhR-activation in the binary mixture.

4.3 AhR-ER cross-talk

To characterize the binary mixture's anti-estrogenic MoA, the present study assessed some of previously proposed cross-talk mechanisms between AhR and ER (Safe and Wormke, 2003). Cells exposed to BNF in combination with E2 displayed a tendency for lower *cyp1a* expression than cells exposed in the absence of E2. Although these differences were not statistically significant, the observed trend suggest a potential weak E2-mediated interference with AhR-mediated *cyp1a* transcription. It has previously been proposed that estrogenic compounds (e.g. 17α-ethinylestradiol (EE2), E2) interfere with the CYP1A transcriptional and protein expression both *in vivo* (Hoffmann et al., 2006; Pajor et al., 1990) and *in vitro* (Gräns et al., 2010; M. Hultman et al., 2015; Mortensen et al., 2006) through AhR-ER cross-talk (Mortensen et al., 2006). Additional cross-talk mechanisms not investigated herein, may cause reduction in ER activation by direct suppression of ER transcription via AhR-ligand binding XRE upstream of ER (Matthews and Gustafsson, 2006). This has previously been described *in vitro* for BNF where exposure of cells to BNF and the AhR/CYP1A-antagonist 8-methoxypsoralen eliminated the BNF-induced reduction of E2-induced Vtg protein expression (Navas and Segner, 2000). Thus, BNF was suggested to exhibit its anti-estrogenic effect through interaction with upstream located XRE of ER-responsive genes (Navas and Segner, 2000).

To further characterize the possible cross-talk mechanisms involved in the anti-estrogenic effects, the common transcription co-factor aryl hydrocarbon receptor translocator protein (ARNT) (Brunnberg et al., 2003; Rüegg et al., 2008) was studied. The ARNT is a dimerization partner for several basic helix-loop-helix (bHLH)- Per-AhR/ARNT-Sim homology sequence (PAS) protein super families (e.g. AhR) (Gu et al., 2000). The present study did not identify a clear effect of the differently acting anti-estrogens on the transcriptional activity of *arnt*. Interestingly, *arnt* was significantly induced in cells exposed to E2 compared to OHT, BNF+OHT (without co-exposure of E2) and the DMSO control (Suppl. Fig. 3), indicative of

its potential role as an ER-α co-activator, as previously suggested by Brunnberg et al. (2003). As gene expression does not always reflect the translated protein and its activity, additional studies should be performed to assess the role of *arnt* as a nuclear receptor co-activator and how this gene and protein function in the AhR-ER cross-talk in fish.

5 Conclusion

The present study showed that the differently acting anti-estrogenic compounds BNF, OHT and a mixture of these all significantly reduced the ER-mediated Vtg protein expression, independent of their specific anti-estrogenic MoA. The compounds caused a similar tendency for reduction of E2-induced genes *er-α*, *vtg-1* and *zrp*, illustrating their potential ability to modulate the activity of both ER-sensitive genes and proteins. The strongest anti-estrogenic effects were, as expected, reported in the mixture of BNF and OHT. The effect of the BNF and OHT mixture on the inhibition of the E2-induced Vtg protein expression agreed well with predictions for additivity. A significant reduction of the E2 induced *vtg-1* transcription by OHT suggested that the anti-estrogenic effect of this compound was mediated by the ER signalling pathway. No clear conclusions on the specific anti-estrogenic MoA for BNF and the mixture of OHT and BNF could be drawn. The present study also assessed the involvement of specific genes involved in putative AhR-ER cross-talk, however none could be clearly associated with the compounds anti-estrogenic MoA and their involvement remains inconclusive. Although the MoAs of the single compounds and the mixture were not completely characterised, the present study has enhanced our knowledge of the combined toxicity mediated by anti-estrogens acting by different MoA.

6 Acknowledgements

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Figures

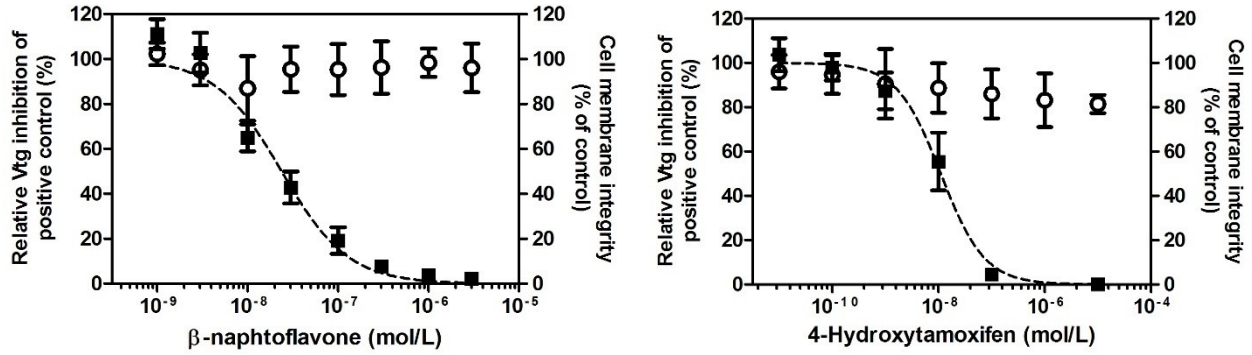


Figure 1. Inhibition of vitellogenin protein expression (left axis) and metabolic activity (right axis) in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes exposed to 17β-estradiol (E2: 6.3E^{-10} mol/L) in combination with 1.0 E^{-9}-3.0 E^{-6} mol/L β-naphtoflavone and 1.0 E^{-12}-1.0 E^{-5} mol/L 4-hydroxytamoxifen for 96h. Data is presented as relative expression of the solvent control (0%) and positive control (30nM E2; 100%) and represent the mean ± standard deviation of 3 individual cell isolations.

Figure 2

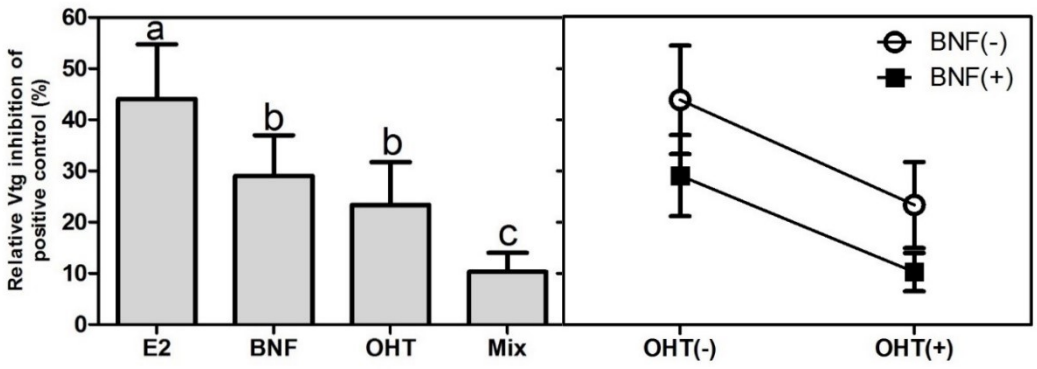


Figure 2. Vitellogenin protein expression (Vtg) in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes exposed to solvent control dimethyl sulfoxide (DMSO) and 17β-estradiol

(E2: $6.3E^{-10}$ mol/L) alone and in combination with β-naphtoflavone (BNF: $2.38E^{-8}$ mol/L), 4-hydroxytamoxifen (OHT: $1.1E^{-8}$ mol/L), and a mixture of these (BNF and OHT) for 96h. Data is presented as relative expression of the DMSO control (0%) and positive control (30nM E2; 100%) and represent the mean of 4 individual cell isolations ± standard deviation. The statistical analysis for the left graph was performed using a one-way ANOVA with a Tukey's post hoc test while the right graph presents a two-way ANOVA using Bonferroni post hoc test. The different letters and * denote treatments which are significantly ($p \leq 0.05$) different from each other in both the one-way and two-way ANOVA, respectively.

Figure 3

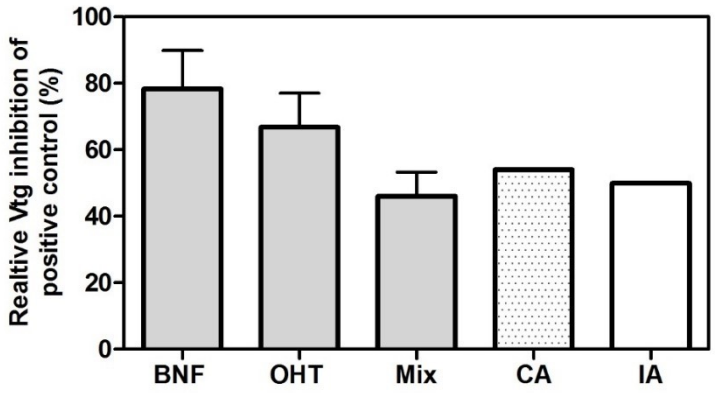


Figure 3. Observed effect of β-naphthoflavone (BNF: $2.38E^{-8}$ mol/L), 4-hydroxytamoxifen (OHT: $1.1E^{-8}$ mol/L) alone and in mixture on the 17β-estradiol (E2, $6.3E^{-10}$mol/L) induced vitellogenin (Vtg) protein expression in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes. Data are normalised between DMSO control (0%) and positive control (E2: $6.3E^{-10}$ mol/L, 100%) and represent the mean and standard deviation (stdev) of 4 individual cell isolations. The predicted effect of the total mixture concentration of BNF and OHT was calculated by the concentration addition (CA) and independent action (IA) prediction model.

Figure 4

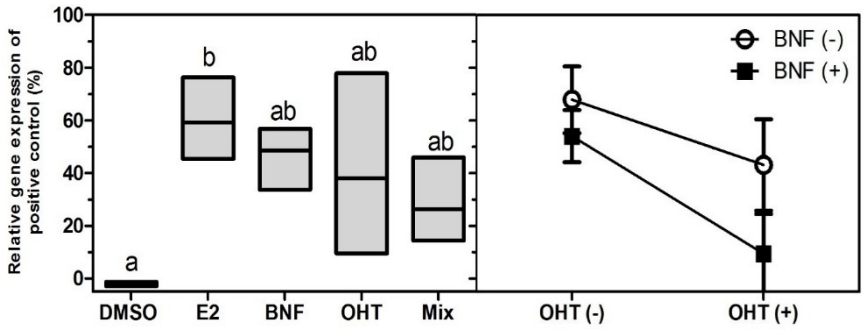


Figure 4. *Estrogen receptor α* (*erα*) transcription in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes after exposure to solvent control dimethyl sulfoxide (DMSO) and $6.3E^{-10}$ mol/L 17β-estradiol (E2), β-naphtoflavone (BNF: $2.38E^{-8}$ mol/L), 4-hydroxytamoxifen (OHT: $1.1E^{-8}$ mol/L) and mixture of these (BNF+OHT) when co-exposed with E2 for 48h. Data represent the mean± standard deviation of 3 individual cell isolations. The statistical analysis for the left graph was performed using a one-way ANOVA with a Tukey's post hoc test while the right graph presents a two-way ANOVA using Bonferroni post hoc test. The different letters and * denote treatments which are significantly ($p \leq 0.05$) different from each other in both the one-way and two-way ANOVA, respectively.

Figure 5

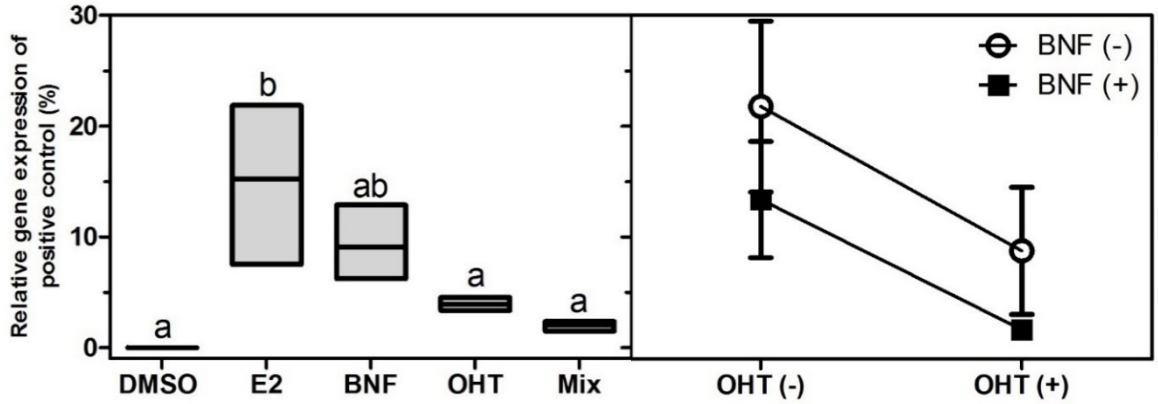


Figure 5. *Vitellogenin-1* (*vtg-1*) gene expression in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes after exposure to solvent control dimethyl sulfoxide (DMSO), $6.3E^{-10}$ mol/L 17β-estradiol (E2), β-naphtoflavone (BNF: $2.38E^{-8}$ mol/L), 4-hydroxytamoxifen (OHT: $1.1E^{-8}$ mol/L) and mixture of these (BNF+OHT) when co-exposed with E2 for 48h. Data represent the mean of 3 individual cell isolations ± standard deviation. The statistical analysis for the left graph was performed using a one-way ANOVA with a Tukey's post hoc test while the right graph presents a two-way ANOVA using Bonferroni post hoc test. The different letters and * denote treatments which are significantly ($p \leq 0.05$) different from each other in both the one-way and two-way ANOVA, respectively.

Figure 6

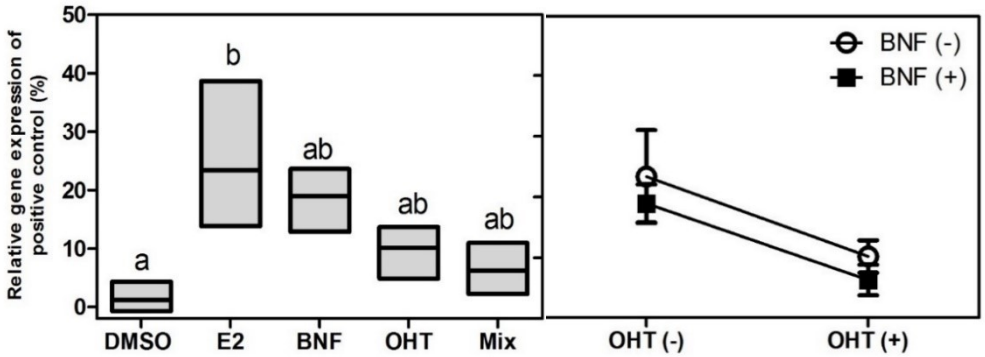


Figure 6. *Eggshell zona radiata protein* (*zrp*) gene expression in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes after exposure to solvent control dimethyl sulfoxide (DMSO), $6.3E^{-10}$ mol/L 17β-estradiol (E2), β-naphtoflavone (BNF: $2.38E^{-8}$ mol/L), 4-hydroxytamoxifen (OHT: $1.1E^{-8}$ mol/L) and mixture of these (BNF+OHT) when co-exposed with E2 for 48h. Data represent the mean of 3 individual cell isolations ± standard deviation. The statistical analysis for the left graph was performed using a one-way ANOVA with a Tukey's post hoc test while the right graph presents a two-way ANOVA using Bonferroni post

hoc test. The different letters and * denote treatments which are significantly ($p \leq 0.05$) different from each other in both the one-way and two-way ANOVA, respectively.

Figure 7

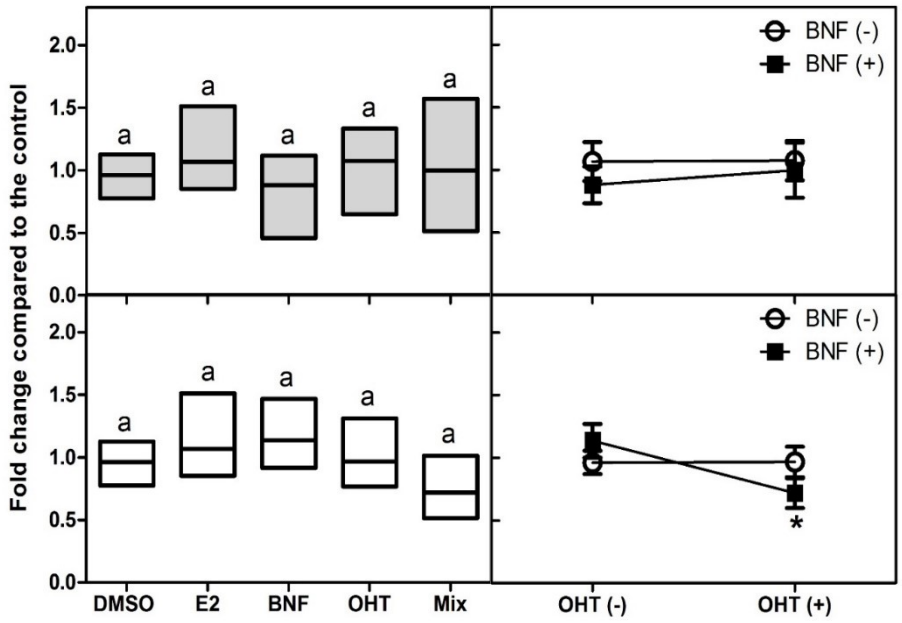


Figure 7. Gene expression of *aryl hydrocarbon receptor 2β* (*ahr2β*) in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes after exposure to solvent control dimethyl sulfoxide (DMSO), $6.3E^{-10}$ mol/L17β-estradiol (E2), β-naphtoflavone (BNF: $2.38E^{-8}$ mol/L), 4-hydroxytamoxifen (OHT: $1.1E^{-8}$ mol/L) and mixture of these (BNF+OHT) in presence (grey boxes) and absence (clear boxes) of E2 for 48h. Data represent the mean of 4 individual cell isolations ± standard deviation. The statistical analysis for the left graph was performed using a one-way ANOVA with a Tukey's post hoc test while the right graph presents a two-way ANOVA using Bonferroni post hoc test. The different letters and * denote treatments which are significantly ($p \leq 0.05$) different from each other in both the one-way and two-way ANOVA, respectively.

Figure 8

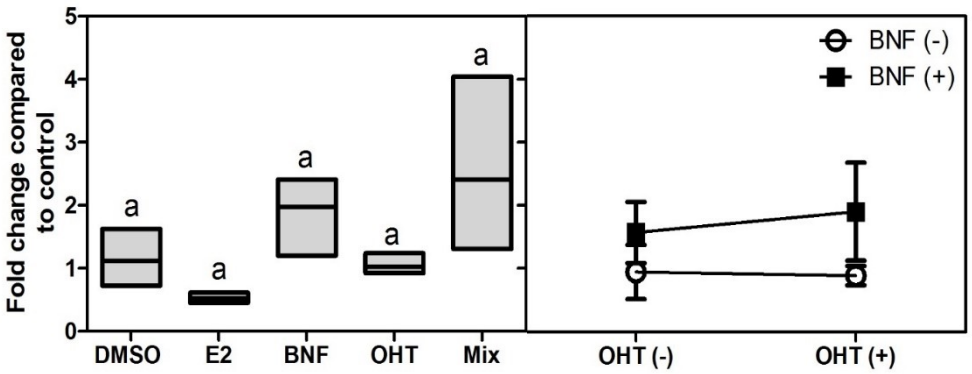


Figure 8. Transcriptional expression of *cytochrome P450 1a* (*cyp1a*) in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes after exposure to solvent control dimethyl sulfoxide (DMSO), $6.3E^{-10}$ mol/L 17β-estradiol (E2), β-naphtoflavone (BNF: $2.38E^{-8}$ mol/L), 4-hydroxytamoxifen (OHT: $1.1E^{-8}$ mol/L) and mixture of these (BNF+OHT) in presence of E2 for 48h. Data represent the mean of 4 individual cell isolations ± standard deviation. The statistical analysis for the left graph was performed using a one-way ANOVA with a Tukey's post hoc test while the right graph presents a two-way ANOVA using Bonferroni post hoc test. The different letters and * denote treatments which are significantly ($p \leq 0.05$) different from each other in both the one-way and two-way ANOVA, respectively.

Tables

Table 1. Experimental design for exposure of β-naphtoflavone (BNF), 4-hydroxytamoxifen (OHT) to rainbow trout (*Oncorhynchus mykiss*) hepatocytes.

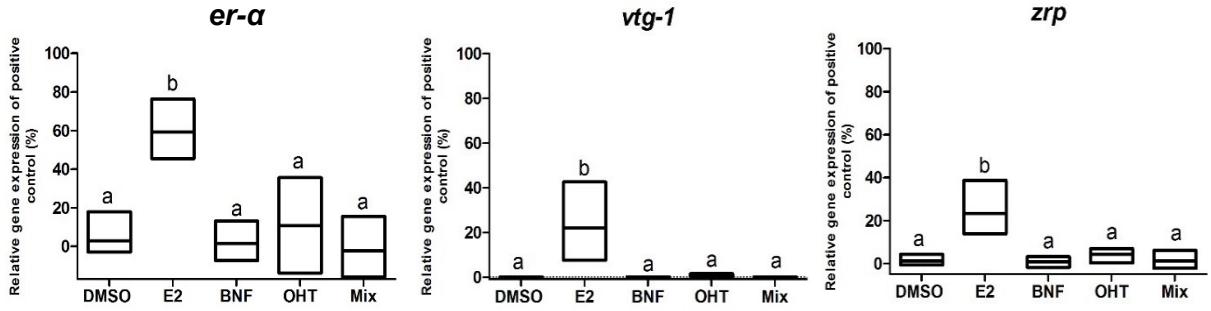
Chemical	**Experiment 1**	**Experiment 2**
E2	EC_{50} ($6.3E^{-10}$ mol/L)*	EC_{50} ($6.3E^{-10}$ mol/L)*
BNF	1.0 E^{-9}-3.0 E^{-6} mol/L	$2.38E^{-8}$ mol/L
OHT	1.0 E^{-12}-$1.0E^{-5}$ mol/L	$1.10E^{-8}$ mol/L
BNF+OHT		$2.38E^{-8}$ (IC_{71}) + $1.10E^{-8}$ (IC_{39})

* Effect concentration measured as vitellogenin protein expression at 50% induction.

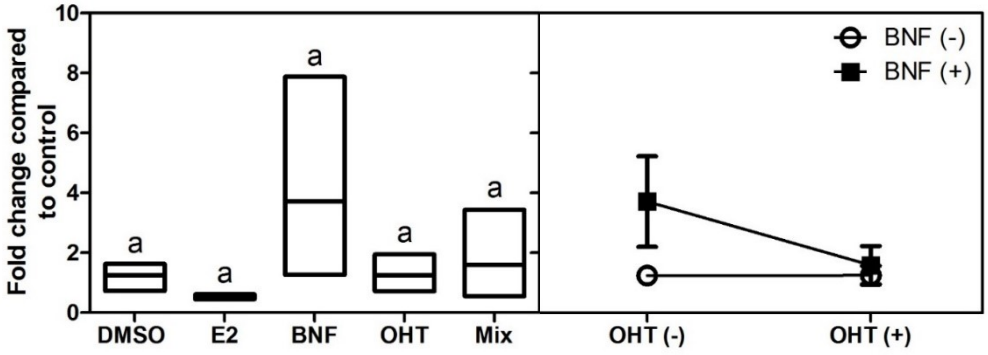
Table 2. Species, gene name, primer sequences, product size, accession numbers (Acc. No.) and analysis protocol used for the qPCR analysis.

Species	Name	Sequence		Amplicon size (bp)	Acc. No.	Annealing Temperature (ºC)	Primer conc. (nM)	Efficiency (100%)	Reference
O. mykiss	ubiquitin	*Forward*	5´-ACAACATCCAGAAAGAGTCCAC-3´	NR	AB036060	55	700	103	Hultman et al., 2015a
		Reverse	5´-AGGCGAGCGTAGCACTTG-3´				700		
O. mykiss	erα	*Forward*	5´-CCCTGCTGGTGACAGAGAGAA-3´	NR	NR	61	270	109	Nagler et al., 2007
		Reverse	5´-ATCCTCCACCACCATTGAGACT-3´				620		
O. mykiss	vtg-1	*Forward*	5´-GAGCTAAGGTCCGCACAATTG-3´	NR	X92804	61	700	105	Celius et al., 2000
		Reverse	5´-GGGAAACAGGGAAAGCTTCAA-3´				700		
S. salar	zrp	*Forward*	TGACGAAGGTCCTCAGGG	113	AF407574	59.4	500	90	Arukwe et al., 2007
		Reverse	AGGGTTTGGGGTTGTGGT				500		
S. salar	ahr2β	*Forward*	5´-GCACCCCCAGGACCAGAGT-3´	96	AY219865	57.6	900	93	Mortensen et al., 2006
		Reverse	5´-GTTGTCCTGGATGACGGC-3´				900		
S. salar	arnt-1	*Forward*	5´-AGAGCAATCCCAGGGTCC-3´	107	DQ367887	57.6	700	93	Mortensen et al., 2006
		Reverse	5´-TGGGAGGGTGATTGAGGA-3´				700		
O. mykiss	cyp1a	*Forward*	5´-TCCTGCCGTTCACCATCCCACACTGCAC-3´	NR	U62797.1	57	700	91	Gräns et al., 2010
		Reverse	5´-AGGATGGCCAAGAAGAGGTAGACCTC-3´				700		

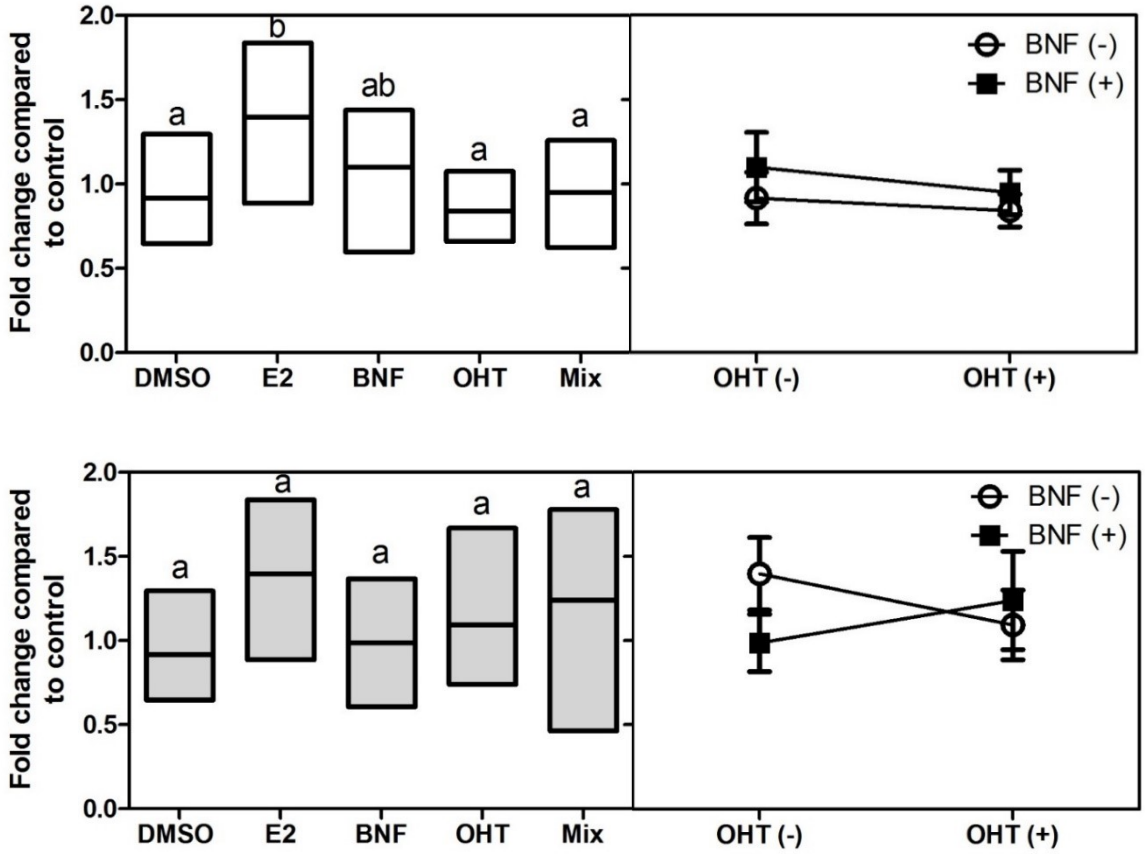
Supplementary information



Supplementary Figure 1. *Gene expression of putative transcripts included in the estrogen receptor signaling (ER) pathway in primary rainbow trout (Oncorhynchus mykiss) hepatocyte after exposure to 6.3E^{-10}M 17β-estradiol (E2), 2.38E^{-8} M β-naphtoflavone (BNF), 1.1E^{-8}M 4-hydroxytamoxifen (OHT) and mixture of these (BNF+OHT) for 48h. Data represent the mean of 3-4 individual cell isolations ± standard deviation. The statistical analysis was performed using a one-way ANOVA with a Tukey's post hoc test ($p \leq 0.05$).*



Supplementary Figure 2. *Gene expression of cytochrome P450 1a (cyp1a) in primary rainbow trout (Oncorhynchus mykiss) hepatocyte after exposure to 6.3E^{-10} mol/L 17β-estradiol (E2), 2.38E^{-8} mol/L β-naphtoflavone (BNF), 1.1E^{-8} mol/L 4-hydroxytamoxifen (OHT) and mixture of these (BNF+OHT) for 48h in absence of 6.3E^{-10} mol/L E2. Data represent the mean of 3 individual cell isolations ± standard deviation. The statistical analysis ($p \leq 0.05$) was performed using a one way-ANOVA with a Tukey's post hoc test (left graph) and a two-way ANOVA with a Bonferroni post hoc test (right graph). Letters denotes statistical significance in the one way-ANOVA, while * denotes statistical significant interaction in the two way-ANOVA.*



Supplementary Figure 3. *Gene expression of aryl hydrocarbon receptor nuclear translocator (arnt) in primary rainbow trout (Oncorhynchus mykiss) hepatocyte after exposure to 6.3E^{-10} mol/L 17β-estradiol (E2), 2.38E^{-8} mol/L β-naphtoflavone (BNF), 1.1E^{-8} mol/L 4-hydroxy-tamoxifen (OHT) and mixture of these (BNF+OHT) for 48h in absence (clear boxes) and co-exposure (filled boxes) with 6.3E^{-10} mol/L E2. Data represent the mean of 3 individual cell isolations ± standard deviation. The statistical analysis ($p \leq 0.05$) was performed using a one-way ANOVA with a Tukey's post hoc test (left graph) and a two way-ANOVA with a Bonferroni post hoc test (right graph). Letters denotes statistical significance in the one way-ANOVA, while * denotes statistical significant interaction in the two way-ANOVA.*

Supplementary Table 1. *Observed and predicted results normalised between DMSO control (0%) and positive control (E2: 6.3E^{-10} mol/L).*

CA – Concentration addition; IA – Independent action; MDR - model deviation ratios

	Experiment 2			
Exposure	*Concentration (mol/L)*	*Predicted effect (% of E2 control)*	*Observed effect (% of E2 at 6.3E^{-10}M)± stdev*	*MDR*
E2	6.3E^{-10}	100		
BNF	2.38E^{-8}	71	71 ± 5.4	
OHT	1.10E^{-8}	39	51 ± 12	
BNF + OHT	2.38E^{-8} + 1.10E^{-8}	38 (CA), 28 (IA)	23 ± 4.7	2.0 (CA) 1.3 (IA)

***Supplementary table 2**. A two-way ANOVA comparing gene expression in factorial treatments exposed to E2, BNF, OHT and BNF+OHT with and without presence of E2. The presented F value represents the ratio of the experimental effect compared to the given 'error', while $p \leq 0.05$ was determined as statistically significant. Significant values are given in bold.*

	F (p)	**Vtg protein**	***er-α***	***vtg-1***	***zrp***	***ahr2β***	***cyp1a***	***arnt***
E2+	BNF	**26.09 (0.002)***	2.428 (0.1702)	**6.455 (0.044)***	2.401 (0.1722)	0.387 (0.5569)	3.753 (0.1008)	0.503 (0.5047)
	OHT	3.171 (0.1253)	**7.577 (0.0332)***	3.002 (0.1339)	1.347 (0.2899)	0.270 (0.6218)	0.054 (0.8236)	0.010 (0.9229)
	Mix	0.099 (0.7627)	0.425 (0.5388)	0.045 (0.8391)	0.6696 (0.4445)	0.068 (0.8035)	0.211 (0.6623)	2.226 (0.1863)
E2-	BNF	NR	1.158 (0.3232)	1.865 (0.0.2210)	1.033 (0.3438)	0.410 (0.5455)	2.798 (0.1454)	4.781 (0.0714)
	OHT	NR	0.073 (0.7962)	2.44 (0.1693)	0.344 (0.5789)	1.768 (0.2319)	1.576 (0.2560)	0.299 (0.6042)
	Mix	NR	0.776 (0.4124)	0.747 (0.4207)	2.40 (0.1723)	**13.06 (0.0112)***	1.604 (0.2523)	0.318 (0.5935)
BNF+	E2	NR	**17.51 (0.0058)***	**10.24 (0.0286)***	**16.13 (0.007)***	0.005 (0.9459)	1.458 (0.2727)	0.322 (0.5909)
	OHT	NR	5.09 (0.0649)	5.078 (0.0651)	3.159 (0.1258)	0.9441 (0.3687)	0.679 (0.4414)	0.041 (0.8460)
	E2+OHT	NR	5.187 (0.063)	4.764 (0.0718)	**6.395 (0.0447)***	2.645 (0.155)	2.608 (0.1575)	1.63 (0.2489)
BNF-	E2	NR	**24.23 (0.0027)***	**11.69 (0.0142)***	**9.991 (0.0195)***	0.878 (0.3849)	0.913 (0.3761)	**9.949 (0.0197)***
	OHT		0.367 (0.5668)	1.631 (0.2488)	0.0001 (0.9917)	0.002 (0.9699)	0.010 (0.9255)	0.754 (0.4185)
	E2+BNF	NR	2.307 (0.1796)	2.047 (0.2025)	1.868 (0.2207)	0.0001 (0.9920)	0.010 (0.9252)	0.9662 (0.3636)
OHT+	E2	NR	**6.909 (0.0391)***	3.664 (0.1041)	1.091 (0.3365)	2.632 (0.1559)	0.003 (0.9591)	1.551 (0.2594)
	BNF	NR	2.323 (0.1783)	2.123 (0.1954)	3.084 (0.1296)	0.724 (0.4276)	1.426 (0.2774)	0.538 (0.4911)
	E2+BNF	NR	0.4139 (0.5438)	1.602 (0.2526)	0.1868 (0.6807)	0.2022 (0.6687)	0.3502 (0.5756)	0.012 (0.9177)
OHT-	E2	NR	**35.97 (0.001)***	**8.481 (0.0269)***	**5.582 (0.0561)***	0.2351 (0.6450)	1.832 (0.2247)	0.554 (0.4848)
	BNF	NR	1.451 (0.2737)	**7.105 (0.0373)***	0.334 (0.5844)	0.004 (0.9537)	4.246 (0.085)	1.408 (0.2803)
	E2+BNF	NR	0.9758 (0.3614)	**6.734 (0.0409)***	1.879 (0.2196)	2.768 (0.1472)	1.512 (0.2649)	**9.687 (0.0208)***

***Abbreviations:** E2 - 17β-estradiol; BNF – β-naphtoflavone; OHT – 4-Hydroxytamoxifen; Mix – BNF+OHT; ahr2β – aryl hydrocarbon receptor 2β; cyp1a – cytochrome P450 1a; er-α – estrogen receptor α; zrp – eggshell zona radiata protein; vtg-1 – vitellogenin 1; Vtg protein – vitellogenin protein; NR – No data reported.*