Profiling of benzophenone derivatives using fish and human estrogen receptor-specific in vitro bioassays

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Abstract

Benzophenone (BP) derivatives, BP1 (2,4-dihydroxybenzophenone), BP2 (2,2',4,4'-tetrahydroxybenzophenone), BP3 (2-hydroxy-4-methoxybenzophenone), and THB (2,4,4'-trihydroxybenzophenone) are UV-absorbing chemicals widely used in pharmaceutical, cosmetics, and industrial applications. Studies on their endocrine disrupting properties have mostly focused on their interaction with human estrogen receptor alpha (hERα), and there has been no comprehensive analysis of their potency in a system allowing comparison between hERα and hERβ activities. The objective of this study was to provide a comprehensive ER activation profile of BP derivatives using ER from human and fish origin in a battery of in vitro tests, *i.e.*, competitive binding, reporter gene based assays, vitellogenin (Vtg) induction in isolated rainbow trout hepatocytes, and proliferation based assays. The ability to induce human androgen receptor (hAR)-mediated reporter gene expression was also examined. All BP derivatives tested except BP3 were full hERα and hERβ agonists (BP2 > THB > BP1) and displayed a stronger activation of hERβ compared with hERα, the opposite effect to that of estradiol (E_2). Unlike E_2, BPs were more active in rainbow trout ERα (rtERα) than in hERα assay. All four BP derivatives showed anti-androgenic activity (THB > BP2 > BP1 > BP3). Overall, the observed anti-androgenic potencies of BP derivatives, together with their proposed greater effect on ERβ versus ERα activation, support further investigation of their role as endocrine disrupters in humans and wildlife.

Key words: Estrogenic activity; Benzophenones (BPs); Receptor binding assay; Reporter gene assay; Cell proliferation assay; Rainbow trout estrogen receptor alpha (rtERα); Human estrogen receptor alpha and beta (hERα and hERβ); Vitellogenin (Vtg); Human androgen receptor (hAR).

Introduction

Over the past few decades, increasing awareness of sunlight-induced damage from ultraviolet (UV) radiations has fuelled the widespread use of topical sunscreen preparations to protect human skin (Eide and Weinstock, 2006). Sun protection products developed by the cosmetics industry contain various so-called "UV screens", *e.g.*, salicylates, dibenzoylmethanes, cinnamates, and benzophenones (BPs), which decrease sunburn by absorbing UVA (400-315 nm) and UVB (315-280 nm) rays (Bielecka-Grzela et al., 2005; Gaspar and Maia Campos, 2006). UV screens are also used to protect scents and colors against UV damage in cosmetic products and against sunlight-induced degradation of manufactured products (Fisher, 1992).

BPs consist of 12 main derivatives, designated BP1 through BP12, as well as other less known BPs, principally used as photostabilizers in cosmetics and as sunscreens in lotions and hair sprays to protect skin and hair from UV irradiation. BP1 (2,4-dihydroxybenzophenone), BP2 (2,2',4,4'-tetrahydroxybenzophenone), and THB (2,4,4'-trihydroxybenzophenone), which consist of two benzene rings joined by a carbonyl group and carrying two, four or three hydroxyl groups, respectively (Fig. 1), are mainly used in personal care products and in plastics used for food packaging, while BP3 (2-hydroxy-4-methoxybenzophenone) is one of the primary UVA/UVB sun-blocking agents in skin care products.

Since topical sunscreens are routinely applied to the skin by a large percentage of the population, human exposure to UV screens *via* dermal absorption is of considerable interest. Thus, the toxicological properties and metabolism of BP3 are well documented. In rats, BP3 is absorbed *via* oral and dermal routes and is excreted in urine and bile (Nakagawa et al., 2000; Nakagawa and Suzuki, 2002). BP3 is also enzymatically converted to at least three intermediates, *i.e.*, BP1, a major intermediate formed by O-demethylation of the parent compound, which is converted to 3HBP (2,3,4-trihydroxybenzophenone) and BP8 (2,2'-dihydroxy-4-methoxybenzophenone) by aromatic hydroxylation (Okereke et al., 1994). Both BP3 and its metabolite BP1 have been detected in human urine after application of commercial products to the skin (Felix et al., 1998; Gonzalez et al., 2006). Schlecht et al. (2008) recently reported that BP2 was metabolized to glucuronide and sulfate conjugates in a dose-response experiment in rats, which received a daily dose of BP2 *per gavage* for 5 days. Maximum serum BP2, BP2-

glucuronide, and BP2-sulfate levels were observed at 30 min after BP2 application, whereas the highest urine concentrations of BP2 and its metabolites were observed at 120 min after treatment. Despite this rapid metabolism, the amount of unconjugated BP2 was sufficient to induce a dose-dependent estrogenic effect in the uterus (Schlecht et al., 2008).

UV screens can enter into the aquatic system directly *via* swimming and bathing by users of these compounds or indirectly *via* wastewater treatment plants. Because most UV screen components are photostable and many are highly lipophilic (Poiger et al., 2004), they are prone to bioaccumulation in biota and wildlife. Unfortunately, only a few UV screens, *e.g.*, OC (octocrylene), 4MBC (4-methylbenzylidene camphor), HMS (homosalate) and BP3, have been investigated in the environment to date. Thus, residues of these UV screens have been detected in lakes and wastewater and in fish (Balmer et al., 2005; Buser et al., 2006). Chemicals released into the environment by UV screens may also reach humans *via* the food chain (Cuderman and Heath, 2007).

Environmental estrogens have been suspected of modulating the endocrine system *via* multiple mechanisms of action and may potentially affect growth, development, and reproduction in wildlife and humans (Damstra et al., 2002; McKinley et al., 2008). Many of the effects of environmental estrogens are mediated by activation of estrogen receptors (ERs), which function as ligand-dependent transcription factors (Tsai et al., 1994; Beato et al., 1995). Both ERs (ERα and ERβ) bind 17β-estradiol (E_2) with high affinity and both bind to estrogen response elements (ERE) in a similar way. However, they differ in amino acid sequence, transcriptional activity, and tissue distribution pattern (Mosselman et al., 1996; Kuiper et al., 1996; Nilsson et al., 2001).

Over the past few years, ER expression has been documented in various vertebrates, and multiple forms of ER have been demonstrated in fish and humans, among other species (Hawkins et al., 2000; Sabo-Attwood, et al., 2004). Although ERs from closely related species exhibit similar binding affinities for endogenous and exogenous estrogens (Tollefsen et al., 2002), large differences in estrogen binding have been demonstrated among receptors from different species (Matthews et al., 2000; Harris et al., 2002). For instance, the ERα from rainbow trout (*Oncorhynchus mykiss*) has a highly divergent amino acid sequence within its ligand binding region (E-domain) and shows a similarity with the hERα of only 60% (Pakdel et al., 1989). Hence, there may be differential ligand binding preferences and/or affinities for endogenous and exogenous estrogens in

fish and humans, among other species (Petit et al., 1995; Matthews et al., 2000; Olsen et al., 2005; Kunz et al., 2006). In this context, BP1, BP2, BP3 or THB have demonstrated in vitro estrogenic activity in MCF-7 human breast cancer cells (Schlumpf et al., 2001; Nakagawa and Suzuki, 2002; Matsumoto, et al., 2005), recombinant cell lines (Yamasaki et al., 2003; Kawamura et al., 2005; Schreurs et al., 2005; Suzuki et al., 2005), and recombinant yeast systems carrying hERα (Miller et al., 2001; Schultz et al., 2000; Morohoshi et al., 2005; Kunz et al., 2006; Kunz and Fent, 2006) or rtERα (Kunz et al., 2006; Kunz and Fent, 2006). Estrogenic activity has also been observed in vivo in rats (Schlumpf et al., 2001; Yamasaki et al., 2003; Seidlová-Wuttke et al., 2004; Jarry et al., 2004; Koda et al., 2005) and fish (Kunz et al., 2006; Weisbrod et al., 2007). Moreover, BPs have also shown in vitro (anti)androgenic activity in recombinant cell lines (Suzuki et al., 2005) and recombinant yeast systems carrying hAR (Kunz and Fent, 2006).

Although the potential activity of BP derivatives *via* hERα has been investigated, much less is known about the interaction of these compounds with hERβ. Moreover, only one study to date, using a recombinant yeast system, has described the estrogenic activity of BP derivatives on rtERα (Kunz et al., 2006). The aim of the present study was to establish a comprehensive profile on the interactions of currently used BP derivatives (BP1, BP2, BP3 and THB) with ERs from fish (rtERα) and human tissues (hERα and hERβ). Various established stable reporter cell lines developed in our laboratory were used for this purpose. ER affinities and estrogenic potencies were evaluated in an array of in vitro test systems. Reporter gene assays were also carried out to investigate the effects of these compounds on the hAR-mediated induction of transcription.

Materials and methods

Chemicals and materials

Culture medium and fetal calf serum (FCS) were obtained from Life Technologies Inc. (Cergy-Pontoise, France). Geneticin and luciferin were purchased from Promega (Charbonnières, France). [^{3}H]-E_2 (41.3 Ci/mmol specific activity) and methyltrienolone (R1881) were purchased from NEN Life Science Products (Paris, France). 17β-estradiol (E_2), 2,4-dihydroxybenzophenone (BP1), 2,2',4,4'-tetrahydroxybenzophenone (BP2), 2-hydroxy-4-methoxybenzophenone (BP3), 2,4,4'-trihydroxybenzophenone (THB), puromycin, and methylthiazolyldiphenyl tetrazolium bromide (MTT) were obtained from Sigma-Aldrich Inc. (St Louis, MO, USA). Stock solutions (10 mM) of E_2, R1881, BP1, BP2, BP3, and THB were prepared in dimethyl sulfoxide (DMSO), and successive dilutions were performed in culture medium. Stock solutions were kept at −20°C and dilution series were freshly prepared before each experiment. All other chemicals were of the highest quality available from commercial sources. All cell culture plastics were obtained from Falcon (Merck Eurolab, Strasbourg, France) except 96-well Cellstar plates, which were obtained from Greiner Labortechnik (Poitiers, France). A MicroBeta Trilux luminometer (EGG Wallac, Turku, Finland) was used to detect luciferase activity in intact cells.

Plasmid constructions

pSG_5-ERα-puro (aa 1-595), pSG_5-ERβ-puro (aa 1-530), pSG_5-AR-puro (aa 1-919), and $pGAL_4$RE-ERE-βGlobin-Luc-SV-Neo were already described (Balaguer et al., 1999; Paris et al., 2002). pSG_5-ΔA/BERα-puro (aa 179-595) was obtained by exchanging the fragment containing ΔA/BERα sequence (digestion *Nde*I-*Bam*HI) from HEG19 vector (gift from P. Chambon, IGBMC, Strasbourg, France) with the *Nde*I-*Bam*HI fragment from pSG_5-puro vector (gift from H. Gronemeyer, IGBMC, Strasbourg, France). pSG_5-ΔA/BERβ-puro (aa 143-530) was cloned by PCR from pSG_5-ERβ-puro using the following primers: 5'-GCGCGCGGATCCACCATGAAGAGGGATGCTCACTTCTGC-3' and 5'-GCGCGCGGATCCTCACTGAGACTGTGGGTTCTG-3'. The PCR product was subcloned into pSG_5-puro vector *via* the *Bam*HI site.

To obtain an expression vector for the rainbow trout receptor (rtER), a plasmid containing the coding short form region of rtERα (aa 46-622) was amplified by PCR

using the following primers: 5'-GCGCGCGGATCCATGTACCCTGAGGAGACACG-3' and 5'-CGCGCGGGATCCTCACGGAATGGGCATCTGG-3' (Pakdel et al., 2000). The 1755 bp *Bam*HI fragment was inserted into the unique *Bam*HI site in pSG_5-puro. Correct cloning was confirmed by restriction enzyme digestion and DNA sequencing.

Reporter cell lines and culture conditions

The stably transfected luciferase reporter MELN cell line was obtained as already described (Balaguer et al., 2001). Briefly, to obtain MELN cells, ERα-positive breast cancer MCF-7 cells were transfected with the estrogen-responsive gene ERE-βGlob-Luc-SV-Neo (Balaguer et al., 1999). MELN cells were cultured in Dulbecco's modified Eagle medium (DMEM) F12 with phenol red, supplemented with 10% FCS, 1% antibiotic (penicillin/streptomycin), and 1 mg/ml G418. Basal luciferase activity in MELN cells was around 15% of maximal activity (100% for 10 nM E_2).

Generation of HELN-ERα, -ERβ, -ΔA/BERα, -ΔA/BERβ, and -rtERα reporter cell lines was performed in two steps (Balaguer et al., 1999; Escande et al., 2006). The estrogen responsive reporter gene was first stably transfected into HeLa cells, generating HELN cell line and, in a second step, these HELN cells were transfected with -ERα, -ERβ, -ΔA/BERα, -ΔA/BERβ, or rtERα plasmid constructs to obtain the HELN-ERα, -ERβ, -ΔA/BERα, -ΔA/BERβ, or -rtERα cell lines, respectively. HELN cells were cultured in DMEM, supplemented with 5% FCS, 1% antibiotic, and 1 mg/ml G418. HELN-ER cells were cultured in DMEM F12 without phenol red, supplemented with 6% dextran-coated charcoal (DCC)-treated FCS (6% DCC-FCS), 1% antibiotic, 1 mg/ml G418, and 0.5 µg/ml puromycin (except for HELN-rtERα cells, which were cultured in DMEM supplemented with 5% FCS). Basal luciferase activity in HELN-ER cells was around 10, 10, 5, 5, and 35% of maximal activity (100% for 10 nM E_2) for -ERα, -ERβ, -ΔA/BERα, -ΔA/BERβ, and -rtERα, respectively.

PALM cells were obtained as already described (Terouanne et al., 2000). Briefly, PC3 cells were co-transfected with an androgen responsive gene, MMTV-Luc-SV-Neo, and an androgen receptor expressing plasmid, pSG_5AR-puro. PALM cells were cultured in Ham's F12 supplemented with 10% FCS, 1 mg/ml G418, and 1 µg/ml puromycin. Basal luciferase in PALM cells was around 10% of maximal activity (100% for 10 nM R1881).

Because of phenol red and FCS estrogenic activity, experiments were performed in test culture medium: DMEM F12 without phenol red, supplemented with 6% DCC-FCS (for MELN, HELN-ERα, -ERβ, -ΔA/BERα, -ΔA/BERβ, and -rtERα cells) or Ham's F12, supplemented with 6% DCC-FCS (for PALM cells) and 1% antibiotic in a 5% CO_2 humidified atmosphere. Experiments were done at 37°C except for HELN-rtERα cells which were incubated at 23°C due to the instability of rtERα at 37°C (Matthews et al., 2001). Test culture medium was used in transactivation assays as well as competitive binding and estrogen-dependent proliferation assays.

Luciferase assay: stable gene expression assay

Reporter cells were seeded at a density of 5×10^4 cells per well in 96-well white opaque tissue culture plates in 150 µl test culture medium. Test compounds were prepared 4× concentrated in the same medium and 50 µl was added per well 8 h after seeding. Cell lines were incubated for 16 h (except for PALM cells, which were incubated for 40 h) with the compounds at 37°C, except for HELN-rtERα cells, which were incubated at 23°C. This lower temperature was used for the luciferase assay because of the thermosensitivity of rtERα (unpublished results). At the end of incubation, the medium containing test compounds was removed and replaced by test culture medium containing 0.3 mM luciferin. At this concentration, luciferin diffused into the cell and produced a stable luminescent signal 5 min later. This signal is approximately 10-fold less intense than the signal obtained after cell lysis but is perfectly stable for several hours. The 96-well plate was then introduced into a Microbeta Wallac luminometer and luminescence was measured in intact living cells for 2 s.

Agonist and antagonist assays

Agonistic activities of hERα, hERβ, hΔA/BERα, hΔA/BERβ, rtERα, and hAR in HELN-derived, MELN, or PALM cells were tested in the presence of increasing concentrations (0.01-10 µM) of BP1, BP2, BP3, and THB. Tests were performed in quadruplicate for each concentration. Results were expressed as a percentage of maximal luciferase activity. Maximal luciferase activity (100%) was obtained in the presence of 10 nM E_2 (for hERs and rtERα) and 10 nM R1881 (for hAR). For each compound, the estrogenic potency corresponding to the concentration yielding half-maximal luciferase activity (EC_{50} value) was calculated. Antagonistic assays for hAR

were performed using a concentration of agonist yielding approximately 85% of maximal luciferase activity. The antagonistic activities of these compounds (tested at 0.01-10 μM) were determined by coincubation with the agonist R1881 at 0.2 nM. Data were expressed as half-maximal inhibitory concentration (IC_{50} value) for each compound tested.

Whole-cell ER competitive binding assays

Briefly, HELN-ERα, -ERβ and -rtERα cells were seeded at a density of 2×10^5 cells per well in 24-well tissue culture plates and grown in test culture medium. After 24 h, HELN-ERα and -ERβ cells were labeled with 0.1 nM [^{3}H]-E_2 (41.3 Ci/mmol specific activity) at 37°C for 3 h in the absence or presence of BP1, BP2, BP3, THB (0.01-10 μM), or unlabelled E_2 (100 nM). For HELN-rtERα, cells were labeled with 0.3 nM [^{3}H]-E_2 at 23°C for 3 h. The final incubation volume was 500 μl, and each well was tested in duplicate. After incubation, unbound material was aspirated and cells washed three times with 500 μl of cold PBS. Then, 250 μl lysis buffer (400 mM NaCl, 25 mM Tris phosphate pH 7.8, 2 mM DTT, 2 mM EDTA, 10% glycerol, 1% triton X-100) was added, and plates were shaken for 5 min. Total cell lysate (200 μl) was mixed with 4 ml of LSC-cocktail (Emulsifier-Safe, Packard BioScience), and [^{3}H] bound radioactivity was liquid scintillation-counted (LS-6000-SC, Beckman-Coulter, Roissy, France). Non-specific binding was determined in the presence of 100 nM unlabeled E_2. Specific binding was calculated by subtracting non-specific binding from total binding. Bound radioactivity values were expressed in disintegrations per minute (dpm). In absence of competitor, specific bound radioactivity was 750-1000 dpm.

Results were plotted as dpm versus concentration of tested compounds. IC_{50} values were defined as the compound concentration required to decrease maximal [^{3}H]-E_2 binding by 50%. Compound selectivity toward hERα, hERβ, or rtERα was evaluated using the relative binding affinity (RBA) to E_2. RBA for each competitor was calculated as the ratio of E_2 to competitor concentration required to reduce specific radiolabeled binding by 50% (ratio of IC_{50} values). The RBA value for E_2 was arbitrarily set at 100.

Lysed cell ER binding assays

In vitro binding assays were performed using hERα and hERβ obtained from HELN-ERα and -ERβ cells, respectively. Briefly, HELN-ERα and -ERβ cells were maintained

in tissue culture flasks (150 cm^2) and grown in test culture medium. Confluent cells were washed with PBS and cells were harvested by trypsinization. After centrifugation at 1,000×*g* for 10 min, supernatants were removed and pellets were stored at −80°C until use. On the day of the competitive binding assay, the pellet (approximately 60×10^6 cells) was suspended in 1 ml of ice cold binding buffer (20 mM Tris-HCL, 5 mM DTT, pH 7.5), and 60 µl of protease inhibitor cocktail (PIC) was added and mixed gently with repeated pipettings. The binding buffer was freshly prepared and left on ice until use. After 30 min of incubation at 4°C, suspensions containing cells were sonicated using a Vibra Cell 72405 sonicator (Bioblock Scientific, Illkirch, France) three times for 5 s bursts each at 30 Hz on ice. The vigorous sonicator treatment lysed about 99% of the cells in the sample. Then, the sonicated material was spun at 13,000×*g* for 10 min at 4°C in a 1.5-ml microcentrifuge tube. The tube was kept on ice throughout the manipulations. After centrifugation, the supernatants (containing hERα or hERβ) were gently gathered and the protein concentration was determined by Bio-Rad protein assay. Solutions of the unlabeled competitors (BP1, BP2, BP3, THB, and E_2) and [^{3}H]-E_2 were prepared 100× concentrated in ethanol. Then, aliquots (90 µl) of the receptor were incubated with 0.1 nM [^{3}H]-E_2 at 4°C for 16 h in the absence or presence of increasing concentrations (0.01-10 µM) of the unlabeled competitors or unlabeled 100 nM E_2 and vehicle (400 µl of binding buffer supplemented with 0.2% of BSA). The final incubation volume was 500 µl, and each well was tested in duplicate. After incubation, unbound steroids were removed by incubation with DCC solution (2% charcoal and 0.2% dextran T_{40}, in binding buffer) on ice, followed by centrifugation at 2,800×*g* for 10 min at 4°C. Finally, aliquots (200 µl) of supernatant were mixed with 4 ml of LSC-cocktail (Emulsifier-Safe, Packard BioScience), and [^{3}H]-bound radioactivity was counted. IC_{50} and RBA values were calculated as described above.

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) toxicity assay

The effect of the BPs on cell viability was assessed with the MTT test using Denizot and Lang's modified technique (1986). In short, cell lines (HELN-derived, MELN, and PALM cells) were seeded at a density of 5×10^4 cells per well in 96-well tissue culture grade for 8 h, followed by treatment with different concentrations (0.01-10 µM) of each compound for a further 16 h. Cells were washed with PBS three times and 100 µl of MTT solution (0.5 mg/ml) was added to each well. After incubation (2 h), viable cells

cleaved the MTT tetrazolium ring into a dark blue formazan reaction product, whereas dead cells remained colorless. The MTT-containing medium was gently removed and DMSO was added to each well. After shaking, the plates were read in absorbance at 540 nm. Additional control consisted of medium alone with no cells. Data were expressed as the average of three wells.

HELN-ERα and -ERβ cell proliferation assays

Briefly, HELN-ERα and -ERβ cells were seeded at a density of 5×10^3 cells per well in 24-well tissue culture plates and grown in test culture medium. Test compounds were added 24 h after seeding. Cell lines were incubated for 10 days at 37°C in the absence or presence of BP1, BP2, BP3, THB (0.01-10 µM), or E_2 (10 nM) with replenishment of ligands in fresh test culture medium every 2 days. The final incubation volume was 400 µl, and each concentration was performed in duplicate. After the incubation period, the medium containing test compounds was removed and replaced by 400 µl of test culture medium containing 0.5 mg/ml MTT. Viable cells cleaved the MTT tetrazolium ring into a dark blue formazan reaction product. After 3 h, reaction was stopped, the MTT-containing medium was gently removed, and formazan salt was solubilized by adding 400 µl of DMSO to each well. Finally, aliquots (50 µl) were transferred to a 96-well plate and the spectrophotometrical absorbance of the formazan product was measured using a microtiter plate reader at 540 nm. The linearity of the MTT assay with cell number was verified prior to cell growth experiments. Results were expressed as percentage of proliferation with respect to the hormone-free control (100%). Data were obtained by dose-response curves plotted as percentage of proliferation versus concentration of the products. The value of IC_{50} was estimated by interpolation of the *x*-axis values corresponding to half the absorbance values of maximal proliferation. IC_{50} values (the concentration of compound that was necessary to obtain 50% of cell proliferation) were then calculated for each test compound.

Vitellogenin (Vtg) assay in primary cultures of rainbow trout hepatocytes (PRTH)

Adult male rainbow trout were obtained from a local hatchery (INRA, Gournay sur Aronde, France) and maintained in tanks with aerated charcoal-filtered tap-water at 15°C. Fish were fed with commercial fish food and acclimatized to laboratory conditions for 2 weeks before use in the experiments. Rainbow trout hepatocytes were

then isolated as previously described (Laville et al., 2004). Freshly isolated hepatocytes were seeded in 96-well plate at a density of 0.5×10^6 cells per well and cultured at 15°C in Leibowitz-15 (L-15) medium supplemented with 1% antibiotics (penicillin/streptomycin). PRTH were left to incubate for 24 h to allow cell attachment and were then exposed to ethanol 0.1% (v/v) (solvent control), E_2 1 µM (positive control), or test chemicals in triplicate for 96 h. After exposure, the culture medium was sampled and stored at –80°C until Vtg determination. Cell viability was checked at the end of incubation by using the MTT assay. Results were normalized to the control value (PRTH treated with ethanol 0.1%) and expressed as percentage of this value.

Vtg quantification was performed using a direct enzyme-linked immunosorbent assay (ELISA) as described by Olsen et al. (2005) with a slight modification. Standard Vtg was purified from female rainbow trout plasma according to the method developed by Brion et al. (2000). The samples or serial dilutions of standard Vtg were centrifuged at 2,000×*g* for 3 min, diluted at 1:2 in coating buffer (0.05 M carbonate/bicarbonate, pH 9.6) and incubated in Maxisorp™ microtiter well overnight at 4°C. Non-specific binding was then blocked with 2% (w/v) of BSA in PBS for 1 h at 37°C. After three washes with PBS-Tween-20 0.05% (v/v), plates were incubated with a monoclonal mouse anti-salmon Vtg antibody (BN-5 diluted 1:2000) for 2 h at 37°C, followed by another washing step and incubation for 2 h at 37°C with an anti-mouse IgG secondary antibody labeled with horseradish peroxidase (1:2,000). After 5 washes with PBS-Tween, enzymatic detection was performed using TMB ELISA substrate, and plates were read at 450 nm using a spectrophotometer microtiter plate reader.

Data analysis

For all assays, each compound was tested at various concentrations in at least three independent experiments and data were expressed as mean ± SD. Individual dose-response curves, in the absence and presence of agonist, were fitted using the sigmoid dose-response function of a graphics and statistics software package (Graph-Pad Prism, version 4.0, 2003, Graph-Pad Software Inc., San Diego, CA, USA). Results are presented as EC_{50} and IC_{50} values. Data were analyzed for significant differences using one-way ANOVA followed by Dunnett's post-comparison test (*vs.* control). Differences were considered statistically significant when $P < 0.05$.

Results

ER in vitro activation by BP derivatives

To evaluate the estrogenic activity of BP1, BP2, BP3 and THB, we first used the MELN cell line, which stably expresses an estrogen-responsive luciferase reporter under the control of endogenous hERα. In this cell line, all four BP derivatives induced luciferase expression in a concentration-response manner (Fig. 2) but with different potencies, in the order BP2 > THB > BP1 > BP3, as indicated by their EC_{50} values (Table 1).

In order to explore whether BPs could act as specific ER modulators, we used stably transfected HELN-ERs cell lines, which allow characterization of ER selectivity (between subtypes and species) and activity (antagonistic, partial or fully agonistic) within the same cellular context (Balaguer et al., 1999; Escande et al., 2006). Dose-response curves in these cells showed substantial differences in assay sensitivity for the natural ER ligand E_2 (Fig. 3). According to the EC_{50} values, the sensitivity of the different cell lines for E_2 decreased in the following order: HELN- ERα > HELN-ERβ > HELN-ΔA/BERα > HELN-rtERα > HELN-ΔA/BERβ.

BP1, BP2, BP3, and THB were first tested for non-specific modulation of luciferase expression on the HELN parental cell line, which contains the same reporter gene as HELN-ERs cells but is devoid of ER. Only BP3 showed non-specific induction of luciferase expression at 10 µM in HELN cells (Fig. 4A). The tests in HELN-ERα yielded similar results to those obtained in MELN cells. BP2 and THB behaved as full hERα agonists, exhibiting full dose-response curves, and BP2 was the most potent agonist (Fig. 4B, Table 1). BP1 induced 60% of maximal luciferase activity at 10 µM, whereas BP3, the least active compound, showed only 22% transactivation at 10 µM concentration, which may be due to non-specific activation as observed in the HELN parental cell line. Interestingly, BP1, BP2, and THB displayed a preference for transactivation of hERβ rather than hERα (Fig.4C, Table 1).

In order to further characterize the agonistic properties of the tested BPs, they were also examined with the HELN-ΔA/BERα and -ΔA/BERβ cell lines. As shown in Table 1, the EC_{50} values for the full ER agonist E_2 in these cell lines differed from those of HELN-ERα and -ERβ cells, in which E_2 displayed a higher potency to transactivate (4.9 to 8.7 fold). When these cells were used to determine hΔA/BERα and hΔA/BERβ agonistic activity of the tested BPs, different EC_{50} values were also observed (Table 1). The full

hERα and hERβ agonists, BP2 and THB, showed a lower potency on deleted hERs (1.5 to 2-fold less potent), similar to E_2. Interestingly, when BP1 was tested, the deletion of A/B domain in hERβ strongly altered its transactivation potency, while the deletion of this domain in hERα affected its potency to a lesser degree (Fig. 4D and 4E). As expected, BP3 was not active on either hΔA/BERα or hΔA/BERβ.

Finally, the ability of these compounds to activate transcription *via* rtERα was examined by using HELN-rtERα cells. As shown in Figure 4F, BP1, BP2, and THB behaved as full rtERα agonists exhibiting full dose-response curves, although the concentration needed for maximal activity varied according to the test compound (1, 3, and 10 μM for BP2, THB, and BP1, respectively). Although the potency of E_2 was lower in the rtERα than in the hERα assay (10-fold less sensitive), the potencies of these compounds were higher with rtERα than with hERα (Table 1). BP2, which was only about 800-fold less potent than E_2, was the most effective agonist followed by THB and BP1 (3,042 and 18,300-fold, respectively). As expected, BP3 was not active.

Effect of BP derivatives on E_2 binding to hERα, hERβ and rtERα

Whole-cell competitive binding assays were performed with HELN-ERα, -ERβ, and -rtERα cells to determine whether the estrogenic effects observed in transactivation assays reflected the abilities of BP1, BP2, BP3, and THB to bind to hERα, hERβ, and rtERα. Table 2 summarizes IC_{50} and RBA values for the two hERs and for rtERα. BP1 inhibited the binding of [^{3}H]-E_2 toward these receptors in a concentration-dependent and competitive manner, although less efficiently than BP2 and THB, which were able to completely displace [^{3}H]-E_2 from hERα, hERβ and rtERα at 10 μM concentration (Fig. 5A, 5B and 5C). BP2 was the most effective compound, with IC_{50} values of 534, 151, and 120 nM for hERα, hERβ, and rtERα, respectively. By contrast, BP3 was inactive and showed no binding affinity for the two hERs or for rtERα, confirming its non-specific effects. Thus, BP1, BP2 and THB showed subtype-selective differences in ligand binding to the two ER subtypes, with higher binding affinities for hERβ than for hERα. These findings indicate that the ability of these compounds to act as ER agonists derived from receptor binding, and the greater affinity for hERβ versus hERα correlated with the preferential agonism of hERβ activity in transactivation assays. It is also noteworthy that E_2 had a higher binding affinity for hERα than for rtERα, while

estrogenic BP derivatives showed an opposite preference for binding to rtERα than to hERα, indicating the different RBA of the two ERs. In fact, the RBAs (Table 2) revealed a 70-fold higher relative affinity of BP2 for rtERα than for hERα.

Next, in vitro binding assays were conducted using the two hERs obtained from lysed HELN-ERα and -ERβ cells in order to explore the possibility that BP2 (the most effective compound) can be metabolized in living HELN-ER cells to compounds that displace [^{3}H]-E_2. Under these conditions, the IC_{50} values of E_2 and as BP2 were very similar to those obtained in whole-cell competitive binding assays (Table 2). BP2 was also able to completely displace [^{3}H]-E_2 from hERα and hERβ. These results suggest that the ability of BP2 to displace [^{3}H]-E_2 from these hERs was not due to metabolism of this compound in living HELN-ER cells.

Proliferative effects of BP derivatives on HELN-ERα and -ERβ cells

We also studied the effects of BP1, BP2, BP3, and THB on cell proliferation to better characterize the estrogenic response of these compounds toward hERα and hERβ. Estrogen agonists inhibit cell proliferation in HELN-ER cells, which serves as an end-point to assess the endogenous cell response to estrogens (Escande et al., paper in preparation). In these cells, E_2 strongly inhibited cell proliferation and IC_{50} values of 0.35 and 0.98 nM were obtained for hERα and hERβ, respectively. BP1, BP2, and THB all inhibited proliferation in a clear dose-dependent manner when BP derivatives were applied to HELN-ERα and -ERβ cells (Fig. 6). All three compounds showed greatest inhibitory effect at 10 μM concentration (20, 55, and 50% for BP1, BP2, and THB, respectively). IC_{50} values in HELN-ERα cells corresponded to 19,242, 1,288 and 2,790 nM, respectively. BP1, BP2, and THB achieved greater cell proliferation inhibition with HELN-ERβ cells, with IC_{50} values of 7,553, 241, and 739 nM, respectively. Again, BP3 had no effect on cell proliferation in either cell line.

Effects of BP derivatives on vitellogenin (Vtg) production by PRTH

Vtg production in PRTH was studied in vitro to assess the effects on a natural endogenous response mediated by rtERα. In these experiments (Fig. 7), basal Vtg production in control cell cultures was not detectable by our ELISA. Maximal Vtg induction by E_2 was obtained at 1 μM, with an EC_{50} of around 100 nM. As can be seen in Figure 7, significant Vtg induction was detected in cultures exposed to BP1, BP2, and

THB, reaching a maximal induction at 30 μM but not in cultures exposed to BP3. Consistent with the data obtained in HELN-rtERα cells, BP2 was the most active chemical to induce Vtg synthesis, followed by THB and BP1. However, these Vtg induction levels were lower than obtained with E_2, with 30 μM BP2 reaching 20% of the maximal response to E_2. At the highest tested concentration (100 μM), cytotoxic events occurred with BP2 and THB (data not shown), explaining the lack of Vtg production at this concentration.

Anti-androgenic potential of BP derivatives

Prompted by reports on the strong anti-androgenic effects of some BP derivatives (Suzuki et al., 2005; Kunz and Fent, 2006), we also examined the androgenic and anti-androgenic activities of BP1, BP2, BP3, and THB using PALM cells. As previously reported (Molina-Molina et al., 2006), the synthetic androgen R1881 exhibited marked androgenic activity in this cell line, with an EC_{50} value of 0.1 nM. With BP derivatives, no androgenic activity was observed in the concentration range of 0.01-10 μM (data not shown). However, hAR antagonistic activity was observed for all four compounds (Fig. 8). THB and BP2 were the most potent AR antagonists, with IC_{50} values of 960 and 1,323 nM, respectively, while BP1 and BP3 were clearly less effective (IC_{50} = 8,554 and >30,000 nM, respectively).

Cell viability

The cytotoxicity of the tested BPs (BP1, BP2, BP3, and THB) was assessed in stably transfected MELN, HELN, and PALM reporter cell lines using the MTT test. In all assays, the tested compounds were devoid of any cytotoxicity (cell survival ranging from 95 to 100%) in the 0.01-10 μM range (data not shown).

Discussion

All BP derivatives investigated in this study, with the exception of BP3, are full hERα and hERβ agonists and activate hERβ more strongly than hERα, the inverse of findings with the natural ligand E_2. All four BPs, including BP3, showed anti-androgenic properties. Importantly, the estrogenicity and anti-androgenicity of BPs were observed in the range of micromolar concentrations described in plasma after normal sunscreen use (Janjua et al., 2004). Moreover, unlike E_2, which was less active in the rtERα than hERα assay, estrogenic BPs showed a higher potency in transactivation assays using rtERα versus hERα when applied at similar concentration ranges to those described in the environment and fish tissues (Balmer et al., 2005).

The estrogenic responses of BP derivatives in HELN-ERα cells were highly similar to those obtained in MELN cells, as previously reported for other ERα ligands (Balaguer et al., 1999). The overall results showed that all BP derivatives, except BP3, exhibited a potent estrogenic activity (BP2 > THB > BP1), with EC_{50} values in the micromolar range. Furthermore, competitive receptor binding assays demonstrated that estrogenicity is mediated *via* binding to hERα. Importantly, all four compounds were also tested in the HELN parental cell line (which does not express hERα), and only BP3 showed non-specific induction of luciferase expression at concentrations above 0.3 μM. Therefore, this apparently weak activity of BP3 might not be due to hERα-specific induction. In contrast, the strong estrogenic activity of BP1, BP2 and THB detected is the result of a direct interaction with the hERα protein, as suggested by binding assays, and not a consequence of a non-specific activation of the basal transcriptional machinery. The estrogenic potencies observed are in agreement with those previously reported by Kawamura et al. (2005) using a mammalian (CHO-K1 cells) reporter gene assay. Similar ranking and EC_{50} values to the present findings were also reported by Suzuki et al. (2005) in a reporter gene assay using MCF-7 cells.

By contrast, Schlumpf et al. (2001) showed that BP3 induced a potent estrogenic response in the MCF-7 cell proliferation assay. This discrepancy with our results may be related to differences in measured end-points (luciferase expression *vs.* cell proliferation) and in the duration of exposure (16 h *vs.* 6 days). In fact, other authors also failed to observe any estrogenicity for BP3 in a recombinant yeast system carrying the hERα (Miller et al., 2001; Kunz et al., 2006) and some found a higher hERα

transactivation activity with BP1 than with BP2 (Kunz et al., 2006). Overall, divergent results have been reported on the in vitro estrogenicity of these compounds, which may be explained by the use of distinct systems (*e.g.*, yeasts *vs.* mammalian cells) with different metabolic and chemical uptake capacities. It was recently reported that BP3 shows a potent anti-estrogenic activity at higher concentrations (Kunz and Fent, 2006), contrasting with our finding of no antagonistic activity for any of the tested BPs in MELN or HELN-ERα cells. Again, discrepancies may result from the different concentration ranges used, *i.e.*, maximum concentration of 10 μM in our study versus substantially higher concentrations (up to 1000 μM) in their investigation. Our selection of a concentration range of 0.01-10 μM took account of the adverse effects (*e.g.*, cytotoxicity) and non-specific effect on luciferase expression observed at higher concentrations and the human and environmentally relevant ranges of concentrations reported for exposure (Janjua et al., 2004).

As demonstrated in other UV screens (Schreurs, et al., 2002; Schlumpf et al., 2004), this study reports, for the first time in some BP derivatives, that BP1, BP2 and THB can strongly stimulate hERβ-mediated gene expression (BP2 > THB > BP1) and show a higher affinity for binding to hERβ than to hERα. The binding affinities of BP1, BP2 and THB for hERβ were consistent with the estrogenic activities defined in the reporter gene assay system. In contrast, a cell-based estrogen reporter assay using HEK293 cells (Schreurs et al., 2002) found that BP3 was able to activate hERβ but at a concentration of 0.1 mM, 10-fold higher than the highest concentration tested in our study. Moreover, Seidlová-Wuttke et al. (2004), utilizing recombinant ERα and ERβ proteins, indicated that BP2 had high binding affinity for both receptor subtypes, although they found no differences in ligand binding between ER subtypes.

Most studies on the estrogenicity of BPs have focused on their interaction with hERα, and there has been much less research on their interaction with hERβ. In fact, mechanisms for ER-mediated gene regulation are complex and depend on the recruitment of tissue-specific co-regulatory factors that differentially affect the interaction of ERs with EREs of different target genes (Klinge, 2001). Thus, the selective binding of BPs with ERα and ERβ may produce differential molecular effects that eventually impact on the physiological response of sensitive cells. ERα and ERβ have markedly different tissue distributions, giving estrogen signaling the function of achieving a balance between the two opposing forces (ERα and ERβ) and their splice

variants (Heldring et al., 2007). These two pathways can be selectively modulated with subtype-selective chemicals. In this regard, it has been suggested that both the nature of ERE and the ERα:ERβ ratio in a given cell or tissue may influence ER-responsive genes after treatment with bisphenol-A, a well characterized endocrine disruptor (Pennie et al., 1998), or after exposure to a metabolite of dietary lignans, enterolactone, which activates ER-mediated transcription in vitro at physiological concentrations with a preference for ERα (Penttinen et al., 2007).

Moreover, the ability of BP derivatives to activate transcription *via* two truncated hERs was examined using HELN-ΔA/BERα and -ΔA/BERβ cell lines. Two activation functions have been described in ER (Tora et al., 1989): the A/B domain, which possesses a ligand-independent activation function (AF-1), and the E domain, which has a ligand-dependent activation function (AF-2). Comparison of the activities toward hERα and hERβ with the truncated hΔA/BERα and hΔA/BERβ provides a powerful model to identify partial ER agonists (requiring ligand-independent AF-1 to induce maximal ER activation), because they share identical sequences except for their N-terminal region A/B domain. Previous studies reported that the agonist activity of some ER ligands was entirely [*i.e.*, OH-tamoxifen, raloxifen for ERα (Pike et al., 1999; Barkhem et al., 1998)] or partially [*i.e.*, ferutinin for ERβ (Ikeda et al., 2002; Escande et al., paper in preparation)] due to AF-1 activity. HELN hΔA/BERα and hΔA/BERβ were used to determine whether BPs display AF-1 dependency. E_2 showed less potency to transactivate ΔA/BERα and ΔA/BERβ, with EC_{50} values of 0.093 nM and 0.58 nM, respectively. BP1, BP2 and THB also showed lower potencies to activate deleted hERs as compared to non-deleted receptors. However, the maximal activity of these compounds is not decreased by deletion of the A/B domain, indicating that BPs are not dependent on AF-1, unlike other selective ER modulators.

Because estrogens inhibit the growth of estrogen receptor-negative breast cancer cells that express a recombinant ER (Garcia et al., 1992; Zajchowski et al., 1993), we also assessed the ability of the tested BPs to inhibit proliferation using HELN-ER cells. Previous studies indicated that estrogen-dependent antiproliferative effects are not limited to ER-negative breast cancer cells, since stable transfection of the ER into Chinese hamster ovary cells (Kushner et al., 1990), rodent fibroblasts (Gaben and Mester, 1991), and a human osteosarcoma cell line (Watts et al., 1989) also resulted in estrogen-dependent growth inhibition. We show here, for the first time, that all three

BPs inhibited cell proliferation in a clear dose-dependent manner (BP2 > THB > BP1) in both cell lines. In addition, this inhibition was higher in HELN-ERβ than in -ERα cells, confirming the higher affinity of BPs for ERβ than for ERα observed in transactivation and competitive binding assays. By contrast, BP3 had no effect on cell proliferation in either cell line, again verifying that this compound is not estrogenic in the range of concentrations tested.

Finally, as reported by others (Kawamura et al., 2003; Suzuki et al., 2005), the key structural requirement for the ERα-mediated estrogenic activity of BP derivatives is a phenolic hydroxyl group. Moreover, the number and position of hydroxyl substituents appear to play a significant role in the estrogenicity of these and similar biphenolic compounds (Rivas, et al., 2002), with compounds hydroxylated at the 2-, 4- and 4'-positions showing highest activity (Suzuki et al., 2005). This tendency was also observed in the present reporter gene, proliferation, and binding assays in MELN, HELN-ERα, and -ERβ cells. Hence, hydroxyl groups may be also a factor in estrogenic activity *via* hERβ, as demonstrated for other compounds, *e.g.*, biphenyl derivatives (Paris et al., 2002).

The lower sensitivity to E_2 of rtERα versus hERα observed in this study is in agreement with previous studies that compared its affinity to human and fish ERs (Le Drean et al., 1995; Matthews et al., 2002). In fact, rtERα and hERα display some differences in the relative and absolute binding affinities of several xenoestrogens, which can be partially attributed to divergences in the amino acid sequences within the ligand binding domain of these ERs (Petit et al., 1995; Pakdel et al., 2000; Matthews et al., 2000; Petit et al., 2000; Le Guével and Pakdel, 2001; Olsen et al., 2005). Various reporter gene assays have shown rtERα to be around 10-fold less sensitive than hERα to E_2 (Le Drean et al., 1995; Matthews et al., 2002). Although the same finding was also observed in in vitro binding assays (Olsen et al., 2005; Matthews et al., 2000), this loss of sensitivity is not systematically found. For instance, some hydroxylated polychlorobiphenyls (PCBs), alkylphenols, and bisphenol A bind to and activate rtERα at equal or lower concentrations than those required to activate hERα (Matthews et al., 2000; Ackermann et al., 2002). In our study, estrogenic BPs showed a higher potency to stimulate rtERα versus hERα in transactivation assays. Absolute sensitivities of rtERα and hERα systems varied very little, suggesting that the main difference between these receptors may be their sensitivity to E_2. BP2 exhibited the most variable potency across species,

with reporter gene EC_{50} values of 1,749 and 161 nM for hERα and rtERα, respectively. Kunz et al. (2006), using a recombinant yeast system carrying the rtERα, also reported the full estrogenic activity of BP1, BP2, and THB but not BP3. However, unlike in the present study, BP1 was found to be the most potent agonist, followed by BP2 and THB. Hence, as for hERα, our rtERα assay is not consistent with these findings. Discrepancies in potencies can most likely be attributed to the different levels of cellular organization in each assay, *i.e.* yeasts versus mammalian cells.

BP derivatives were tested in a more elaborate biological system using rainbow trout hepatocytes, one of the main estrogen (E_2)-target cells in fish, in which most of the xenobiotic biotransformation capacities are contained in the liver (Pesonen and Andersson, 1991). In the trout hepatocyte primary culture system, the regulation of the expression of several genes, especially those of Vtg and ER, has been extensively studied and was found to be remarkably similar to that observed in vivo (Flouriot et al., 1995). Hence, Vtg gene expression in our trout hepatocyte cultures was used as a biological marker for the exposure to estrogenic compounds. In this bioassay, the increase in hepatic Vtg levels confirmed that BP1, BP2, and THB have estrogenic activity. However, much higher concentrations of BP1, BP2 and THB were needed to induce Vtg in comparison to the concentrations required to activate rtERα in the reporter gene assay. Reasons for these discrepancies between the two in vitro assays are not clear, although it has been suggested that variations in metabolic capacities may influence the estrogenicity of chemicals (Beresford et al., 2000; Fang et al., 2000). However, dose-dependent increases in Vtg were observed in fish (fathead minnows) exposed to BP1 or BP2 but not to BP3 (Kunz et al., 2006), confirming the present results.

Since several UV screens with estrogenic activity have also been shown to possess (anti)androgenic activity (Ma et al., 2003; Suzuki et al., 2005; Kunz and Fent, 2006), we investigated the possible (anti)androgenic activity of BP derivates. All four BPs exhibited a potent anti-androgenic activity in PALM cells, though the activity markedly varied depending on their chemical structure (THB > BP2 > BP1 > BP3). Hence, BPs substituted with hydroxyl groups at 2-, 4-, and 4'-positions showed the highest anti-androgenic activity, confirming data previously described by Suzuki et al. (2005). However, other authors (Ma et al., 2003; Schreurs et al., 2005) reported lower IC_{50} values for the anti-androgenic activity of BP3 in a cell-based androgen reporter assay

using U2-OS and MDA-kb2 cells. An explanation for these differences may be that both cell lines metabolize BP3 and thereby activate this compound, whereas the PALM cells used in our assay are probably less efficient in biotransforming the inactive parent compound. Moreover, a study of some commonly used UV screens (including BP1, BP2 and BP3) in a recombinant yeast system found BP1 to be a more potent anti-androgenic UV screen compared with BP2 and BP3, which showed a similar potency between them (Kunz and Fent, 2006). In addition, BP2 showed a potent androgenic activity in the hAR assay. These results differ from the present findings. However, in a reporter gene assay using NIH-3T3 cells, Suzuki et al. (2005) reported the same ranking and similar IC_{50} values for all four compounds, which showed no agonistic activity, in agreement with our study. As in in vitro estrogenic assays, divergent results have also been reported in yeast and mammalian cell systems.

Anti-androgenic activities of BPs are of concern because in vivo studies reported that THB exhibited anti-androgenic activity in a Hershberger assay using F344 rats (Suzuki et al., 2005). In this study, we demonstrated that all four BP derivatives show anti-androgenic activity. The IC_{50} values found for BP2 and THB were in the same range as that reported for the well-known fungicide vinclozolin (Molina-Molina et al., 2006). Exposure of fish to anti-androgens has been associated with gonadal changes (induction of intersex), reduced spermatogenesis, demasculinization, and reduced sperm counts (Bayley et al., 2003; Kiparissis et al., 2003). Hence, the presence of anti-androgenic BPs in the aquatic environment might present a plausible alternative explanation for alterations in fish reproductive functions that have been associated with the presence of xenoestrogens (Sumpter, 2005). Further studies are warranted to elucidate the relevance of the anti-androgenicity of BP derivatives to changes in fish reproductive functions.

In conclusion, the endocrine disrupting activity of the BP derivatives investigated in this study may pose a risk to humans and wildlife. Although the potency of these compounds as hormone agonists or antagonists is low compared with natural ligands, their ability to interfere with signaling pathways at different levels might contribute to their harmful biological effects. Furthermore, since living organisms are simultaneously exposed to a large variety of xenobiotics, potential additive and/or synergistic effects must be taken into consideration (Heneweer et al., 2005; Charles et al., 2007), underscoring the need for further investigation into the role of BP derivatives used in cosmetic and health care products.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Mis en forme : Anglais (Royaume-Uni)

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References

Mis en forme : Allemand (Allemagne)

Ackermann, G. E., Brombacher, E., Fent, K., 2002. Development of a fish reporter gene system for the assessment of estrogenic compounds and sewage treatment plant effluents. Environ. Toxicol. Chem. 21, 1864-1875.

Balaguer, P., François, P., Comunale, F., Fenet, H., Boussioux, A. M., Pons, M., Nicolas, J. C., Casellas, C., 1999. Reporter cell lines to study the estrogenic effects of xenoestrógenos. Sci. Total Environ. 233, 47-56.

Balaguer, P., Boussioux, A. M., Demirpence, E., Nicolas, J. C., 2001. Reporter cell lines are useful tools for monitoring biological activity of nuclear receptor ligands. Luminescence. 16, 153-158.

Balmer, M. E., Buser, H. R., Muller, M. D., Poiger, T., 2005. Occurrence of some organic UV filters in wastewater, in surface waters, and in fish from Swiss Lakes. Environ. Sci. Technol. 39, 953-962.

Barkhem, T., Carlsson, B., Nilsson, Y., Enmark, E., Gustafsson, J., Nilsson, S., 1998. Differential response of estrogen receptor alpha and estrogen receptor beta to partial estrogen agonists/antagonists. Mol. Pharmcol. 54, 105-112.

Bayley, M., Larsen, P. F., Bækgaard, H., Baatrup, E., 2003. The effects of vinclozolin, and anti-androgenic fungicide, on male guppy secondary sex characters and reproductive success. Biol. Reprod. 69, 1951-1956.

Beato, M., Herrlich, P., Schutz, G., 1995. Steroid hormone receptors: many actors in search of a plot. Cell. 83, 851-857.

Beresford, N., Routledge, E. J., Harris, C. A., Sumpter, J. P., 2000. Issues arising when interpreting results from an in vitro assay for estrogenic activity. Toxicol. Appl. Pharmacol. 162, 22-33.

Bielecka-Grzela, S., Klimowicz, A., Zaluga, E., Zejmo, M., 2005. Protection of the skin from harmful ultraviolet radiation. Ann. Acad. Med. Stetin. 51, 33-37.

Blair, R. M., Fang, H., Branham, W. S., Hass, B. S., Dial, S.L., Moland, C. L., Tong, W., Shi, L., Perkins, R., Sheehan, D. M., 2000. The estrogen receptor relative binding affinities of 188 natural and xenochemicals: structural diversity of ligands. Toxicol. Sci. 54,138-153.

Brion, F., Rogerieux, F., Noury, P., Migeon, B., Flammarion, P., Thybaud, E., Porcher, J. M., 2000. Two-step purification method of vitellogenin from three teleost fish species: rainbow trout (*Oncorhynchus mykiss*), gudgeon (*Gobio gobio*) and chub (*Leuciscus cephalus*). J. Chromatogr. B. Biomed. Sci. Appl. 737, 3-12.

Mis en forme : Français (France)

Mis en forme : Français (France)

Mis en forme : Français (France)

Mis en forme : Allemand (Allemagne)

Buser, H. R., Balmer, M. E., Schmid, P., Kohler, M., 2006. Occurrence of UV filters 4-methylbenzylidene camphor and octocrylene in fish from various Swiss rivers with inputs from wastewater treatment plants. Environ. Sci. Technol. 40, 1427-1431.

Charles, G: D., Gennings, C., Tornesi, B., Kan, H. L., Zacharewski, T. R., Bhaskar-Gollapudi, B., Carney, E. W., 2007. Analysis of the interaction of phytoestrogens and synthetic chemicals: an in vitro/in vivo comparison. Toxicol. Appl. Pharmacol. 218, 280-288.

Cuderman, P., Heath, E. 2007. Determination of UV filters and antimicrobial agents in environmental water samples. Anal. Bioanal. Chem. 387, 1343-1350.

Damstra, T., Page, S. W., Herrman, J. L., Meredith, T., 2002. Persistent organic pollutants: potential health effects? J. Epidemiol. Community Health. 56, 824-825.

Denizot, F., Lang, R., 1986. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J. Immunol. Methods. 89, 271-277.

Eide, M. J., Weinstock, M. A., 2006. Public health challenges in sun protection. Dermatol. Clin. 24, 119-124.

Escande, A., Pillon, A., Servant, N., Cravedi, J. P., Larrea, F., Muhn, P., Nicolas, J. C., Cavailles, V., Balaguer, P., 2006. Evaluation of ligand selectivity using reporter cell lines stably expressing estrogen receptor alpha or beta. Biochem. Pharmacol. 71, 1459-1469.

Fang, H., Tong, W., Perkins, R., Soto, A., Prechtl, N. V., Sheehan, D. M., 2000. Quantitative comparisons of in vitro assays for estrogenic activities. Environ. Health Perspect. 108, 723-729.

Felix, T., Hall, B. J., Brodbelt, J. S., 1998. Determination of benzophenone-3 and metabolites in water and human urine by solid-phase microextraction and quadrupole ion trap GC-MS. Anal. Chim. Acta. 371, 195-203.

Fisher, A. A., 1992. Sunscreen dermatitis: Part III--The benzophenones. Cutis. 50, 331-332.

Flouriot, G., Pakdel, F., Ducouret, B., Valotaire, Y., 1995. Influence of xenobiotics on rainbow trout liver estrogen receptor and vitellogenin gene expression. J. Mol. Endocrinol. 15, 143-151.

Garcia, M., Derocq, D., Freiss, G., Rochefort, H., 1992. Activation of estrogen receptor transfected into a receptor-negative breast cancer cell line decreases the metastatic and invasive potential of the cells. Proc. Natl. Acad. Sci. USA. 89, 11538-11542.

Mis en forme : Français (France)

Gaspar, L. R., and Maia Campos, P. M., 2006. Evaluation of the photostability of different UV filter combinations in a sunscreen. Int. J. Pharm. 307, 123-128.

Gonzalez, H., Farbrot, A., Larko, O., Wennberg, A. M., 2006. Percutaneous absorption of the sunscreen benzophenone-3 after repeated whole-body applications, with and without ultraviolet irradiation. Br. J. Dermatol.154, 337-340.

Harris, H. A., Bapat, A. R., Gonder, D. S., Frail, D. E., 2002. The ligand binding profiles of estrogen receptors alpha and beta are species dependent. Steroids. 67, 379-384.

Gaben, A. M., and Mester, J., 1991. BALB/c mouse 3T3 fibroblasts expressing human estrogen receptor: effect of estradiol on cell growth. Biochem. Biophys. Res. Commun. 776, 1473-1481.

Hawkins, M. B., Thornton, J. W., Crews, D., Skipper, J. K., Dotte, A., Thomas, P., 2000. Identification of a third distinct estrogen receptor and reclassification of estrogen receptors in teleosts. Proc. Natl. Acad. Sci. USA. 97, 10751-10756.

Heldring, N., Pike, A., Andersson, S., Matthews, J., Cheng, G., Hartman, J., Tujague, M., Ström, A., Treuter, E., Warner, M., Gustafsson, J. A., 2007. Estrogen receptors: how do they signal and what are their targets. Physiol Rev. 87, 905-931.

Heneweer, M., Muse, M., van den Berg, M., Sanderson, J. T., 2005. Additive estrogenic effects of mixtures of frequently used UV filters on pS2-gene transcription in MCF-7 cells. Toxicol. Appl. Pharmacol. 208, 170-177.

Ikeda, K., Arao, Y., Otsuka, H., Nomoto, S., Horiguchi, H., Kato, S., Kayama, F., 2002. Terpenoids found in the umbelliferae family act as agonists/antagonists for ER(alpha) and ERbeta: differential transcription activity between ferutinine-liganded ER(alpha) and ERbeta. Biochem. Biophys. Res. Commun. 291, 354-360.

Janjua, N. R., Mogensen, B., Andersson, A. M., Petersen, J. H., Henriksen, M., Skakkebaek, N. E., Wulf, H. C., 2004. Systemic absorption of the sunscreens benzophenone-3, octyl-Methoxycinnamate, and 3-(4-methyl-menzylidene) camphor after whole-body topical application and reproductive hormone levels in humans. J. Invest. Dermatol. 123, 57-61.

Jarry, H., Christoffel, J., Rimoldi, G., Koch, L., Wuttke, W., 2004. Multi-organic endocrine disrupting activity of the UV screen benzophenone 2 (BP2) in ovariectomized adult rats after 5 days treatment. Toxicology. 205, 87-93.

Mis en forme : Allemand (Allemagne)

Mis en forme : Allemand (Allemagne)

Mis en forme : Allemand (Allemagne)

Kawamura, Y., Mutsuga, M., Kato, T., Lida, M., Tanamoto, K., 2005. Estrogenic and anti-androgenic activities of benzophenones in human estrogen and androgen receptor mediated mammalian reporter gene assays. J. Health Sci. 51, 48-54.

Kiparissis, Y., Metcalfe, T. L., Balch, G. C., Metcalfe, C. D., 2003. Effects of antiandrogens, vinclozolin and cyproterone acetate on gonadal development in the Japanese medaka (*Oryzias latipes*). Aquat. Toxicol. 63, 391-403.

Klinge, C. M., 2001. Estrogen receptor interaction with estrogen response elements. Nucleic Acids Res. 29, 2905-2019.

Koda, T., Umezu, T., Kamata, R., Morohoshi, K., ohta, T., Morita, M., 2005. Uterotrophic effects of benzophenone derivatives and a p-hydroxybenzoate used in ultraviolet screens. Environ. Res. 98, 40-45.

Kuiper, G. G., Enmark, E., Pelto-Huikko, M., Nilsson, S., Gustafsson, J. A., 1996. Cloning of a novel receptor expressed in rat prostate and ovary. Proc. Natl. Acad. Sci. USA. 93, 5925-5930.

Kuiper, G. G., Lemmen, J. G., Carlsson, B., Corton, J. C., Safe, S. H., van der Saag, P. T., van der Burg, B., Gustafsson, J. A., 1998. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology. 139, 4252-4263.

Kunz, P. Y., Galicia, H. F., Fent, K., 2006. Comparison of in vitro and in vivo estrogenic activity of UV filters in fish. Toxicol. Sci. 90, 349-361.

Kunz, P. Y., Fent, K., 2006. Multiple hormonal activities of UV filters and comparison of in vivo and in vitro estrogenic activity of ethyl-4-aminobenzoate in fish. Aquat. Toxicol. 79, 305-324.

Kushner, P. J., Hort, E., Shine, J., Baxter, J. D., Greene, G. L., 1990. Construction of cell lines that express high levels of the human estrogen receptor and are killed by estrogens. Mol. Endocrinol. 4, 1465-1473.

Laville, N., Aït-Aïssa, S., Gomez, E., Casellas, C., Porcher, J. M., 2004. Effects of human pharmaceuticals on cytotoxicity, EROD activity and ROS production in fish hepatocytes. Toxicology. 196, 41-55.

Le Drean, Y., L. Kern, L., Pakdel, F., Valotaire, Y., 1995. Rainbow trout estrogen receptor presents an equal specificity but a differential sensitivity for estrogens than human estrogen receptor, Mol. Cell. Endocrinol. 109, 27-35.

Le Guével, R., and Pakdel, F., 2001. Streamlined beta-galactosidase assay for analysis of recombinant yeast response to estrogens. BioTechniques. 30, 1000-1004.

Mis en forme : Français (France)

Ma, R., Cotton, B., Lichtensteiger, W., Schlumpf, M., 2003. UV filters with antagonistic action at androgen receptors in the MDA-kb2 cell transcriptional-activation assay. Toxicol. Sci. 74, 43-50.

McKinley, R., Plant, J. A., Bell, J. N. B., Voulvoulis, N., 2008. Endocrine disrupting pesticides: Implications for risk assessment. Environ. Int. 34, 168-183.

Matsumoto, H., Adachi, S., Suzuki, Y., 2005. Estrogenic activity of ultraviolet absorbers and the related compounds. Yakugaku Zasshi. 125, 643-652.

Matthews, J. B., Celius, T., Halgren, R., Zacharewski, T. Z., 2000. Differential estrogen receptor binding of estrogenic substances: a species comparison. J. Steroid Biochem. Mol. Bio. 74, 223-234.

Matthews, J. B., Clemons, J. H., Zacharewski T. R., 2001. Reciprocal mutagenesis between human alpha (L349, M528) and rainbow trout (M317, I496) estrogen receptor residues demonstrates their importance in ligand binding and gene expression at different temperatures. Mol. Cell Endocrinol. 183, 127-139.

Matthews, J. B., Feruck, K. C., Celius, T., Huang, Y. W., Fong, C. J., Zacharewski, T. R., 2002. Ability of structurally diverse natural products and synthetic chemicals to induce gene expression mediated by estrogen receptors from various species. J. Steroid. Biochem. Mol. Bio. 82, 181-194.

Miller, D., Wheals, B. B., Beresford, N., Sumpter, J.P., 2001. Estrogenic activity of phenolic additives determined by an in vitro yeast bioassay. Environ. Health Perspect. 109, 133-138.

Molina-Molina, J. M., Hillenweck, A., Jouanin, I., Zalko, D., Cravedi, J. P., Fernandez, M. F., Pillon, A., Nicolas, J. C., Olea, N., Balaguer, P., 2006. Steroid receptor profiling of vinclozolin and its primary metabolites. Toxicol. Appl. Pharmacol. 216, 44-54.

Morohoshi, K., Yamamoto, H., Kamata, R., Shiraishi, F., Koda, T., Morita, M., 2005. Estrogenic activity of 37 components of commercial sunscreen lotions evaluated by in vitro assays. Toxicol. In Vitro. 19, 457-469.

Mosselman, S., Polman, J., Dijkema, R., 1996. ER beta: identification and characterization of a novel human estrogen receptor. FEBS Lett. 392, 49-53.

Nakagawa, Y., Suzuki, T., 2002. Metabolism of 2-hydroxy-4-methoxybenzophenone in isolated rat hepatocytes and xenoestrogenic effects of its metabolites on MCF-7 human breast cancer cells. Chem. Biol. Interact. 139, 115-128.

Nilsson, S., Makela, S., Treuter, F., Tujague, M., Thomsen, J., Andersson, G., Enmark, E., Pettersson, K., Warner, M., Gustafsson, J. A., 2001. Mechanisms of estrogen action. Physiol. Rev. 81, 1535-1565.

Okereke, C. S., Abdel-Rhaman, M. S., Friedman, M. A., 1994. Disposition of benzophenone-3 after dermal administration in male rats. Toxicol. Lett. 73, 113-122.

Olsen, C. M., Meussen-Elholm, E. T., Hongslo, J. K., Stenersen, J., Tollfsen, K. E., 2005. Estrogenic effects of environmental chemicals: an interspecies comparison. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 141, 267-274.

Pakdel, F., Le Guellec, C., Vaillant, C., Le Roux, M. G., Valotaire, Y., 1989. Identification and estrogen induction of two estrogen receptors (ER) messenger ribonucleic acids in the rainbow trout liver: sequence homology with other ERs. Mol. Endocrinol. 3, 44-51.

Pakdel, F., Métivier, R., Flouriot, G., Valotaire, Y., 2000. Two estrogen receptor (ER) isoforms with different estrogen dependencies are generated from the trout ER gene. Endocrinology. 141, 571-580.

Paris, F., Balaguer, P., Terouanne, B., Servant, N., Lacoste, C., Cravedi, J. P., Nicolas, J. C., Sultan C., 2002. Phenylphenols, biphenols, bisphenol-A and 4-tert-octylphenol exhibit alpha and beta estrogen activities and anti-androgen activity in reporter cell lines. Mol. Cell. Endocrinol. 193, 43-49.

Pennie, W. D., Aldridge, T. C., Brooks, A. N., 1998. Differential activation by xenoestrogens of ER alpha and ER beta when linked to different response elements. J. Endocrinol. 158, 11-14.

Penttinen, P., Jaehrling, J., Damdimopoulos, A. E., Inzunza, J., Lemmen, J. G., van der Saag, P., Pettersson, K., Gauglitz, G., Mäkelä, S., Pongratz, I., 2007. Diet-derived polyphenol metabolite enterolactone is a tissue-specific estrogen receptor activator. Endocrinology. 148, 4875-4886.

Pesonen, M., Andersson, T., 1991. Characterization and induction of xenobiotic metabolizing enzyme activities in a primary culture of rainbow trout hepatocytes. Xenobiotica. 21, 461-471.

Petit, F., Valotaire, Y., Pakdel, F., 1995. Differential functional activities of rainbow trout and human estrogen receptors expressed in the yeast Saccharomyces cerevisiae. Eur. J. Biochem. 233, 584-592.

Mis en forme : Allemand (Allemagne)

Mis en forme : Allemand (Allemagne)

Mis en forme : Français (France)

Mis en forme : Français (France)

Mis en forme : Français (France)

Petit, F. G., Valotaire, Y., Pakdel, F., 2000. The analysis of chimeric human/rainbow trout estrogen receptors reveals amino acid residues outside of P- and D-boxes important for the transactivation function. Nucleic Acids Res. 28, 2634-2642.

Poiger, T., Buser, H. R., Balmer, M. E., Bergqvist, P. A., Muller, M. D., 2004. Occurrence of UV filter compounds from sunscreens in surface waters: regional mass balance in two Swiss lakes. Chemosphere. 55, 951-963.

Rivas, A., Lacroix, M., Olea-Serrano, F., Laíos, I., Leclercq, G., Olea, N., 2002. Estrogenic effect of a series of bisphenol analogues on gene and protein expression in MCF-7 breast cancer cells. J. Steroid Biochem. Mol. Biol. 82, 45-53.

Sabo-Attwood, T., Kroll, K. J., Denslow, N. D., 2004. Differential expression of largemouth bass (*Micropterus salmoides*) estrogen receptor isotypes alpha, beta, and gamma by estradiol. Mol. Cell Endocrinol. 218, 107-118.

Schlecht, C., Holger, K., Frauendorf, H., Wuttke, W., Jarry, H., 2008. Pharmacokinetics and metabolism of benzophenone 2 in the rat. Toxicology. 245, 11-17.

Schlumpf, M., Jarry, H., Wuttke, W., Ma, R., Lichtensteiger, W., 2004. Estrogenic activity and estrogen receptor beta binding of the UV filter 3-benzylidene camphor. Comparison with 4-methylbenzylidene camphor. Toxicology. 199, 109-120.

Schlumpf, M., Cotton, B., Conscience, M., Haller, V., Steinman, B., Lichtensteiger, W., 2001. In vitro and in vivo estrogenicity of UV screens. Environ. Health Perspect. 109, 239-244.

Schreurs, R., Lanser, P., Seinen, W., van der Burg, B., 2002. Estrogenic activity of UV filters determined by an in vitro reporter gene assay and an in vivo transgenic zebrafish assay. Arch. Toxicol. 76, 257-261.

Schreurs, R. H., Sonneveld, E., Jansen, J. H., Seinen, W., van der Burg, B., 2005. Interaction of polycyclic musks and UV filters with the estrogen receptor (ER), androgen receptor (AR), and progesterone receptor (PR) in reporter gene bioassays. Toxicol. Sci. 83, 264-272.

Schultz, T. W., Seward, J. R., Sinks, G. D., 2000. Estrogenicity of benzophenones evaluated with a recombinant yeast assay: comparison of experimental and rules-based predicted activity. Environ. Toxicol. Chem. 19, 301-304.

Seidlová-Wuttke, D., Jarry, H., Wuttke, W., 2004. Pure estrogenic effect of benzophenone-2 (BP2) but not of bisphenol A (BPA) and dibutylphtalate (DBP) in uterus, vagina and bone. Toxicology. 205, 103-112.

Mis en forme : Allemand (Allemagne)

Mis en forme : Allemand (Allemagne)

Mis en forme : Allemand (Allemagne)

Sumpter, J. P., 2005. Endocrine disrupters in the aquatic environment: An overview. Acta Hydrochim. Hydrobiol. 33, 9-16.

Suzuki, T., Kitamura, S., Khota, R., Sugihara, K., Fujimoto, N., Ohta, S., 2005. Estrogenic and antiandrogenic activities of 17 benzophenone derivatives used as UV stabilizers and sunscreens. Toxicol. Appl. Pharmacol. 203, 9-17.

Terouanne, B., Tahiri, B., Georget, V., Belon, C., Poujol, N., Avances, C., Orio, F. J., Balaguer, P., Sultan, C., 2000. A stable prostatic bioluminescent cell line to investigate androgen and anti-androgen effects. Mol. Cell. Endocrinol. 160, 39-49.

Tollefsen, K. E., Mathisen, R., Stenersen, J., 2002. Estrogen mimics bind with similar affinity and specificity to the hepatic estrogen receptor in Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). Gen. Comp. Endocrinol. 126, 14-22.

Tora, L., White, J., Brou, C., Tasset, D., Webster, N., Scheer, E., Chambon, P., 1989. The human estrogen receptor has two independent nonacidic transcriptional activation functions. Cell. 59, 477-487.

Tsai, M. J., O'Malley, B. W., 1994. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu. Rev. Biochem. 63, 451-486.

Watts, C. K. W., Parker, M. G., King, R. J. B., 1989. Stable transfection of the oestrogen receptor gene into a human osteosarcoma cell line. J. Steroid Biochem. 34, 483-490.

Weisbrod, C. J., Kunz, P. Y., Zenker, A. K., Fent, K, 2007. Effects of the UV filter benzophenone-2 on reproduction in fish. Toxicol. Appl. Pharmacol. 225, 255-266.

Yamasaki, K., Takeyoshi, M., Sawaki, M., Imatanaka, N., Shinoda, K., Takatsuki, M., 2003. Immature rat uterotrophic assay of 18 chemicals and Hershberger assay of 30 chemicals. Toxicology. 183, 93-115.

Yamasaki, K., Takeyoshi, M., Yakabe, Y., Sawaki, M., Takatsuki, M., 2003. Comparison of the reporter gene assay for ER-alpha antagonists with the immature rat uterotrophic assay of 10 chemicals. Toxicol. Lett. 142, 119-131.

Zajchowski, D. A., Sager, R. and Webster, L., 1993. Estrogen inhibits the growth of estrogen receptor-negative, but not estrogen receptor-positive, human mammary epithelial cells expressing a recombinant estrogen receptor. Cancer Res. 53, 5004-5011.

Mis en forme : Français (France)

Mis en forme : Allemand (Allemagne)

Legends

Figure 1: Chemical structures of benzophenone (BP) and derivatives.

Figure 2: Induction of luciferase activity in MELN cells by BP derivatives. MELN cells were treated with BP1, BP2, BP3, and THB for 16 h at the indicated concentrations. The maximal luciferase activity (100%) was obtained with 10 nM E_2. Results are expressed as a percentage of maximal E_2 induction. Values were the mean ± SD from three separate experiments. *$p< 0.05$ and **$p< 0.01$ (versus 0.1% ethanol used as a control).

Figure 3: Dose-response curves of E_2 in HELN-ERα, -ERβ, -ΔA/BERα, -ΔA/BERβ, and -rtERα cells. Cell lines were incubated for 16 h at 37°C (except for HELN-rtERα cells, which were incubated at 23°C) in the presence of E_2 at the indicated concentrations. The maximal luciferase activity (100%) was obtained with 10 nM E_2. Results are expressed as a percentage of maximal E_2 induction. Values were the mean ± SD from three separate experiments.

Figure 4: Dose-response curves of BP1, BP2, BP3, and THB in HELN, HELN-ERα, -ERβ, -ΔA/BERα, -ΔA/BERβ, and -rtERα cells. Cells were treated with these chemicals for 16 h at the indicated concentrations. The maximal luciferase activity (100%) was obtained with 0.1% ethanol (HELN) or 10 nM E_2 (HELN- ERα, -ERβ, -ΔA/BERα, -ΔA/BERβ, and -rtERα). Values were the mean ± SD from three separate experiments. *$p< 0.05$ and **$p< 0.01$ (versus 0.1% ethanol used as a control).

Figure 5: Competition inhibition of [^{3}H]-E_2 binding to hERα, hERβ and rtERα by BP derivatives. HELN-ERα, -ERβ and -rtERα cells were incubated with different concentrations (0.01-10 μM) of BP1, BP2, BP3 and THB in presence of 0.1 or 0.3 nM [^{3}H]-E_2. Values were the mean ± SD from three separate experiments. *$p< 0.05$ and **$p< 0.01$ (versus 0.1 or 0.3 nM [^{3}H]-E_2).

Figure 6: Proliferative response of HELN-ERα and -ERβ cells. Dose-response curves to E_2, BP1, BP2 and THB. Cells were treated with indicated compounds for 10 days (with replenishment of ligands in fresh medium every 2 days) at the indicated concentrations.

Data are expressed as percentage with respect to the hormone-free control (100%). Values were the mean ± SD from three separate experiments. *p< 0.05 and **p< 0.01 (versus 0.1% ethanol used as a control).

Figure 7: Effects of E_2, BP1, BP2, BP3, and THB on vitellogenin production by primary cultures of male rainbow trout hepatocytes. After 24 hours of plating, cells were incubated with indicated compounds for 4 days at 15°C. Values were the mean ± SD from three separate experiments.

Figure 8: Induction of luciferase activity in PALM cells by BP derivatives. PALM cells were treated with 0.2 nM R1881 in the presence of increasing concentrations of BP1, BP2, BP3, and THB for 40 h. Maximal luciferase activity (100%) was obtained with 10 nM R1881. Values were the mean ± SD from three separate experiments. *p< 0.05 and **p< 0.01 (versus R1881 0.2 nM).