Selective Activation of Zebrafish Estrogen Receptor Subtypes by Chemicals by Using Stable Reporter Gene Assay Developed in a Zebrafish Liver Cell Line

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Over the past few decades, a growing number of aquatic chemical contaminants have been shown to act as endocrine disruptor compounds (EDCs) (Hotchkiss et al., 2008). EDCs have the ability to interfere with the endocrine system through multiple mechanisms of action, leading to disturb essential physiological processes such as normal sexual development and reproduction of exposed aquatic species. One major mechanism of action of EDCs involves their interaction with specific nuclear receptors such as estrogen receptors (ERs). ERs function as ligand-dependent transcription factors. After agonist ligand binding, the migration of liganded ERs to specific palindromic DNA sequences, called estrogen response elements (ERE), allows the recruitment of transcriptional cofactors and further transcription of ER-regulated genes.

The number of environmental chemical contaminants suspected to act as endocrine disruptor compounds by interacting with estrogen receptor (ER) signaling pathway has been continuously increasing. To study such interaction, the use of stable reporter gene assays is relevant, but species-specific in vitro screening assays are still lacking to address hazard assessment of estrogenic chemicals in aquatic vertebrates. Here, we describe the development of stable reporter gene assays based on stable expression of subtypes of zebrafish ER (zfERα, zfERβ1, and zfERβ2) coupled to estrogen response element–driven luciferase in a zebrafish liver (ZFL) cell line. The three established cell models, named ZELH-zfERα, ZELH-zfERβ1, and ZELH-zfERβ2, expressed stable and significant basal luciferase signal, which was induced by 17β-estradiol (E2) in a sensitive and dose-response manner at EC50s of 0.2, 0.03, and 0.05nM, respectively. In addition, E2 significantly altered cell proliferation in ZELH-zfERα and ZELH-zfERβ2 cells, but not in parental ZFL and ZELH-zfERβ1 cells, suggesting a functionality of these two receptors to modulate endogenous gene expression in the transfected clones. The screening of various xenosterogens from different classes in the three models resulted in different luciferase response patterns. Natural and synthetic estrogens and 1,1,1-trichloro-2-(2 chlorophenyl)-2-(4-chlorophenyl)ethane were active at lower concentrations in ZELH-zfERβ1 and ZELH-zfERβ2 than in ZELH-zfERα cells, whereas genistein and zearalenone metabolites as well as three benzophenone derivatives preferentially activated zfERα. Altogether, the newly established models provide specific and convenient in vitro tool for comparative assessment of zfERs selective activation by chemicals within ZFL cell context.

Key Words: ZFL cells; zebrafish estrogen receptor subtypes; in vitro screening assay.
subtypes from the same species but also between species, yielding significant interspecies differences in ER affinity for chemicals (Matthews et al., 2000; Pakdel et al., 1990).

To understand and to evaluate impact of xenoestrogens on ER-signaling pathway, it is necessary to develop cell-based transcription assay systems that could reflect different cellular contexts and/or different model species. The use of in vitro assays based on reporter gene driven by ERE has proven useful and relevant screening tool to address the large number of chemicals yet needed to be tested for their estrogenic potential. Several stable reporter gene assays based on human ERα activation have been developed in different cell contexts and successfully used to characterize estrogenic potency of chemicals (Balagué et al., 1999; Legler et al., 1999; Wilson et al., 2004). Conversely, a very limited number of in vitro stable reporter gene assays derived from nonmammalian species have been developed. In fish, stable reporter gene assays include expression of rainbow trout ERα (rERα) in human and fish cells (Ackermann et al., 2002; Cosnefroy et al., 2009; Molina-Molina et al., 2008). These studies stressed the need to take into account both the species origin of studied receptor and the cell context in hazard assessment of estrogenic chemicals in fish. However, the current lack of species-specific in vitro screening assay may represent an important gap in hazard assessment of EDCs for aquatic organisms and notably for fish (Hotchkiss et al., 2008).

Small fish including zebrafish (Danio rerio) are increasingly being used as model species to study in vivo effects of EDCs (Bron et al., 2004; Segner, 2009; Vosges et al., 2010). In addition, some in vitro models based on transient transfection of zebrafish ERs (zfERs) have been developed using human cell lines as host cells (Legler et al., 2002; Le Page et al., 2006; Sassi-Messai et al., 2009), but to our knowledge, no stable system using zebrafish cells has been published so far. In zebrafish, three zfER subtypes (zfERα, zfERβ1, and zfERβ2) are present, conversely to mammals where two ER subtypes are expressed (Hawkins and Thomas, 2004; Menuet et al., 2002; Mosselman et al., 1996). zfERs are differently expressed and regulated in reproductive tissue like gonads, liver, as well as in brain. In adult liver, E2 induces zfERα expression while it had no effect on zfERβ2 and repressed zfERβ1 expression (Menuet et al., 2002). Moreover, both zfERα and zfERβ2 upregulated zfERα expression after E2 exposure, whereas zfERβ1 had no effect on this expression (Menuet et al., 2004). These studies suggested that the different forms of zfERs have partially distinct and nonredundant functions. Hence, in a perspective of developing fish in vitro assays, it is essential to take into account all zfER subtypes in the assessment of chemical estrogenicity in zebrafish.

The aim of this study was to develop a new in vitro reporter gene assay that is specific of this model species, i.e., based on expression of zebrafish ERs in a zebrafish hepatic cell line. For this purpose, a two-step stable transfection strategy was used. First, we stably transfected an ERE-luciferase construct in the zebrafish liver cell line (ZFL), yielding a ZELH (ZFL-ERE-Luciferase-Hygromycin) clone. Then, zfERs subtypes were stably transfected within ZELH cells, yielding three stable reporter cell lines that were further characterized toward both a panel of known ER ligands and endogenous ER-mediated response (cell proliferation and gene expression).

**MATERIALS AND METHODS**

**Chemicals, cell culture and molecular biology reagents.** 17β-estradiol (E2), 17α-ethinylestradiol (EE2), estrone (E1), estriol (E3), diethylstilbestrol (DES), hexestrol (HEX), genistein (Gen), α-zearalenol (α-Zee), β-zearalenol (β-Zee), and δ-zearalanol (δ-Zea), 4-tert-octylphenol (4OP), bisphenol A (BPA), 1,1,1-trichloro-2-(2-chlorophenyl)-2-(4-chlorophenyl)ethane (octyl-DDT), 2,4-dihydroxybenzophenone (BP1), 2,2′,4,4′-tetrahydroxybenzophenone (BP2), 2-hydroxy-4-methoxybenzophenone (BP3), and 2,4,4′-trihydroxybenzophenone (THB) were purchased from Sigma-Aldrich (France). Stock solutions of chemicals were prepared in dimethyl sulfoxide (DMSO) at 10mM and stored at −20°C. Fresh dilutions of test chemicals were prepared before each experiment. Leibovitz 15 culture medium (L15), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), fetal calf serum (FCS), epidermal growth factor (EGF), G418, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), RNAlater, and D-luciferin were purchased from Sigma-Aldrich (Quentin Fallavier). Insulin, hygromycin B, and sodium bicarbonate were from Dominique Dutscher (Brumath, France), and F-12 nutrient mixture (Ham’s F12) powder, Dulbecco’s Modified Eagle Medium High Glucose (DMEM HG) powder, penicillin, and streptomycin were purchased from Gibco (France), TRIZol reagent was purchased from Invitrogen (France), SYBR Green Master Mix from Qiagen (France), Mouse Moloney leukemia virus reverse transcriptase (MMLV-RT), random hexamers, deoxyribonucleotide triphosphate (dNTPs), RNasin ribonuclease inhibitor, and MMLV-RT buffer were obtained from Promega (France).

**Plasmids.** The construction of ERE-bGlob-Luc-SVhygro plasmid that encodes for the luciferase reporter gene and the hygromycin resistance gene has been described previously (Balagué et al., 1999). The Topo-pcDNA3 (Invitrogen, San Diego, CA) derived expression vectors containing the coding regions of each zebrafish ER complementary DNA (cDNA) (zfERα, zfERβ1, and zfERβ2) and the neomycin resistance gene have also been described previously (Menuet et al., 2002).

**ZFL cell line, cell culture conditions and transfection.** The ZFL cell line, obtained from the American Type Culture Collection (ATCC CRL-2643), is derived from normal adult ZFL (Ghosh et al., 1994). Zebrafish liver cells were routinely cultured under humidified air atmosphere at 28°C in L15, D-MEM, Ham’s-F12 (LDF) medium containing 50% of L15, 35% of DMEM HG, and 15% of Ham’s F12 with 0.15 g/l sodium bicarbonate, 15mM HEPES, 0.01 mg/ml insulin, 50 ng/ml EGF, and 50 U/ml penicillin and streptomycin antibiotics and supplemented with 5% vol/vol FCS.

For stable transfection experiments, ZFL cells were plated to 60% of confluence in LDF-FCS media. In order to compare expression of the three different zfERs in the same clonal context, we performed the transfection in two subsequent steps, using jetPEI (Ozyme, Saint-Quentin-en-Yvelines, France) as a transfection reagent according to the manufacturer’s instructions. First, ZFL cells were transfected with the ERE-bGlob-Luc-SVhygro. After 24 h, transfection reagent was removed, and cells were allowed to recover for 24 h before addition of 0.35 μg/ml hygromycin B as a selecting antibiotic in LDF-FCS medium. Medium was renewed every day during 1 month before first clones were isolated under luminescent camera and amplified. Six clones were selected as the most luminescent ones and were named ZELH for ZFL-ERE-Luc-Hygromycin. On the second step, six selected ZELH clones were transfected with zfERα and then selected using 3 mg/ml G418 in LDF medium 5% of
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dextran-coated charcoal treated serum (DCC) to establish the ZELH-zfERα cell line. Culture medium was renewed every day before first clones were isolated and tested for luciferase induction by E2. The best responsive clones were selected based on both EC50 values and luciferase induction factors of E2. The initial ZELH clone, which led to the most E2-responsive ZELH-zfERα cells, was selected and used to establish stably transfected ZELH-zfERβ1 and ZELH-zfERβ2 cell lines, by using same procedure. The resulting ZELH cell line expressing ERE-βGlob-Luc-SVhygro was routinely cultured in LDF-FCS medium supplemented with hygromycin B (0.35 µg/ml). The ZELH-zfERs cell lines expressing both ERE-βGlob-Luc-SVhygro and zfERs were cultured in LDF supplemented with 5% DCC, hygromycin B (0.35 µg/ml), and G418 (5 µg/ml).

Chemical exposure and luciferase induction assay. Cells were seeded on 96-well plates at 25,000 cells per well for ZELH-zfERα and ZELH-zfERβ1 cell lines and at 50,000 cells per well for ZELH and ZELH-zfERβ2 cell lines in phenol red free LDF-DCC medium and left to adhere for 24 h before chemical exposure. Cells were exposed to test chemicals for 72 h at 28°C. Solvent content was always 0.1% vol/vol in the culture medium. Luciferase activity was then determined in living cells after rinsing step with 150 µl of PBS and addition of 50 µl of D-luciferin 0.3mM in LDF-DCC medium without phenol red. After 5 min allowing a stabilization of the luminescent signal, luminescence counts were determined in a microplate luminometer (µBeta, Wallac). Results were expressed as percentage of maximal luciferase induced by E2, the reference ligand.

Effect of 17β-estradiol on cell proliferation using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay. Cells were seeded at 20,000 cells per well in 24-well plates in LDF-DCC medium. Twenty-four hours after seeding, cells were exposed for 10 days to a range of concentration of the model estrogen E2. Solvent (DMSO) concentration was always 0.1% vol/vol in the culture medium. Two-step cycles 15 s at 95°C and 1 min at 60°C. Transcription concentration was quantified against a standard curve established using a range of dilutions of target DNA fragments (10−3–10−11 µg/µl). PCR reactions were realized in duplicates in two independent experiments. Each target DNA fragment, i.e., zfERα, zfERβ1, zfERβ2, vtg1, and vtg2, were analyzed in each sample. Results were analyzed using Realplex software 2.2 and were expressed as concentration of target DNA fragment in transfected ZELH-zfERs cell lines relative to that in the parental ZFL wild type cell line.

RESULTS

Differential Expression and Activation of zfER Subtypes by E2

Establishment of zfERs-luciferase zebrafish cell lines was obtained after double stable transfection of ZFL cells, first with the ERE-βGlob-Luc-SV-hygro plasmid to yield the ZELH (ZFL ERE-βGlob-Luc-SV-hygro) clone and then with zfERs expressing plasmids into the ZELH clone. This allowed us to assess reporter gene expression and the modulation of its expression by zfER subtypes into the same clonal context, i.e., ZELH cells. As seen in Figure 1, the luminescent signal was almost similar in ZELH and ZELH-derived cell lines under normal conditions (i.e., the absence of E2) hence suggesting a stable expression of the reporter gene, which was found to be stable over more than 50 passages in the ZELH-zfERα cell line (data not shown). In addition, E2 was unable to induce luciferase in ZELH cells suggesting that no functional endogenous zfER was present in the parental cell line. In zfERs expressing cell lines, maximal luciferase activity induced by E2 was higher in ZELH-zfERα and ZELH-zfERβ2 cells than in ZELH-zfERβ1 cells (Fig. 1).
The established clones were further characterized toward their responsiveness to the reference estrogen E2. Typical dose-response curves for luciferase by E2 in the different cell lines are shown in Figure 2. In these cells, E2 had higher potency on zfERb1 and zfERb2 than on zfERA, with EC50 of 0.027 nM, 0.051 nM, and 0.20 nM, respectively (Table 1). For a comparative purpose, dose-response curves after transient cotransfection of ZFL cells were also established and showed that almost identical patterns to stable transfection were obtained in terms of both effective concentrations of E2 and maximal luciferase induction factors (Fig. 2).

Finally, to further characterize these new cell lines, the expression of zfERs messenger RNA (mRNA) was assessed in recombinant and parental ZFL cell line. The results showed a strong expression of zfERα and zfERβ2 in ZELH-zfERα and ZELH-zfERβ2 cells, respectively, about 20,000-fold over the levels found in the parental ZFL cell line (Fig. 3). Unexpectedly, the expression level of zfERβ1 in ZELH-zfERβ1 cell line was very low, about twofold to fivefold that one found in ZFL cells.

**E2 Modulates Cell Proliferation of zfER-Expressing ZFL Cells**

To further characterize the established cells, we assessed whether the expression of zfERs in ZFL cells could have affected any endogenous cell response that is controlled by estrogens in vivo context. Because cell proliferation has been shown to be negatively affected in mammalian cell lines de novo transfected by ER (Garcia et al., 1992; Molina-Molina et al., 2008), we investigated such effect of E2 on ZELH-zfERs cell lines. We observed a concentration-dependent decrease of cell proliferation for both ZELH-zfERα and ZELH-zfERβ2 cells, whereas no significant effect was observed with ZELH-zfERβ1 cells (Fig. 4). The IC50 of E2 were 0.4 nM and 0.05 nM for ZELH-zfERα and ZELH-zfERβ2, respectively. These data

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**TABLE 1**

Comparison of EC50 Values of E2 on zfERα, zfERβ1, and zfERβ2 in Transient and Stable Transfection Experiments Using ZFL Cells

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Stable transfection EC50 (nM)</th>
<th>Transient transfection EC50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>zfERα</td>
<td>0.20 ± 0.05</td>
<td>0.46 ± 0.13</td>
</tr>
<tr>
<td>zfERβ1</td>
<td>0.03 ± 0.01</td>
<td>0.026 ± 0.018</td>
</tr>
<tr>
<td>zfERβ2</td>
<td>0.05 ± 0.02</td>
<td>0.037 ± 0.019</td>
</tr>
</tbody>
</table>

*Note.* Results are means ± SD of n = 14–15 for stable transfection and n = 3 for transient transfection.

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**FIG. 2.** Concentration-dependent luciferase induction in response to 17β-estradiol (E2) in ZFL cells either transiently (solid motif/dash line) or stably (open motif/plain line) transfected with zfER subtypes and ERE→Glob-Luc-SV-hygro. Luciferase activity is expressed as percentage of maximal luciferase induction by E2 10 nM in ZELH-zfERα cells and E2 3 nM in ZELH-zfERβ1 and ZELH-zfERβ2 cells. Results are means of triplicates ± SD and are representative of three (transient) or 14–15 (stable) independent experiments.

**FIG. 3.** Expression of zfERα, zfERβ1, and zfERβ2 mRNA in stably transfected ZELH-zfERs and parental ZFL cell lines as detected by real-time PCR. Results are expressed as relative levels of zfER mRNA level in stably transfected cells over that measured in parental ZFL cell line. Results (means ± SD) are representative of two independent experiments.

**FIG. 4.** Effect of E2 on cell proliferation in ZELH-zfERα, ZELH-zfERβ1, and ZELH-zfERβ2 cell lines, as determined by MTT coloration test. Results are expressed as % of DMSO-treated cells means ± SD of triplicates and are representative of three independent experiments; *Statistically different from DMSO control (p < 0.05).
suggested that the ER recombinant expression of zfERα and zfERβ2 in ZFL cells could affect endogenous response to estrogens, likely through modulation of ER-dependent genes involved in cell proliferation and/or through cross talk with other signaling pathways involved in cell proliferation.

Luciferase Induction by Different ER Ligands in Reporter Zebrafish Cell Lines

To characterize the potential of established cells as in vitro tools to assess estrogenic chemicals, we tested a panel ER ligands that belong to different chemical classes, including natural (E2, E1, and E3) and synthetic estrogens (EE2, HEX, and DES), a phytoestrogen (Gen), mycoestrogens (α-Zea, β-Zee), as well as industrial chemicals (BP1, BP2, BP3, THB, BPA, o,p’-DDT, and 4tOP). All tested compounds induced luciferase in a concentration-dependent manner in the three ZELH-zfERα cell lines, but some differences in terms of both EC50 (Table 2) and maximal induction (Figs. 5, 6, and 7) could be observed. In addition, among all test compounds, only Gen weakly induced luciferase activity (by 20% over control at 10 μM) in ZELH cells, which do not express functional ER (data not shown). This suggested that unspecific activation of luciferase could not be suspected in our experiments.

Natural and synthetic estrogens acted as full agonists on the three cell lines. E2 and EE2 exerted very similar activation whereas E1 was about threefold to fivefold more active than E3 noticed that E1 and E3 were equipotent in ZELH-zfERα, whereas E1 was about threefold to fivefold more active than E3 in the two ZELH-zfERβ cells.

Concerning zearalenone metabolites, well-described ER ligand: DES > EH > E3, whereas in ZELH-zfERα cells, DES was the most active chemical: DES > EE2 > E2 > HEX > E3 ≥ E1. It was also noticed that E1 and E3 were equipotent in ZELH-zfERα, whereas E1 was about threefold to fivefold more active than E3 in the two ZELH-zfERβ cells (Table 2).

The phytoestrogen genistein (Gen) behaved as a full zFER agonist as it elicited maximal transactivation on the three cell lines with a slightly higher affinity for ZELH-zfERα (Fig. 5C, Table 2). Concerning zearalenone metabolites, well-described ER ligand mycoestrogens, different transactivation patterns were noted depending on the zFER subtype. α-Zea induces 100% of transactivation on ZELH-zfERα (Fig. 5C) and ZELH-zfERβ (Fig. 7C), whereas it induced only 70% of transactivation in ZELH-zfERβ1 (Fig. 6C). Conversely, α-Zea and β-Zee behaved as partial zFERS agonists in all cell models except for β-Zee in ZELH-zfERβ1 cells, where it induced maximal response, but at a high concentration. In the three models, α-Zea and α-Zee were the most active compounds in terms of EC50. Based on their REP, our results showed that tested myco- and phytoestrogens have higher potency for α than for β ER subtypes (Table 2).

Industrial compounds (BPA, o,p’-DDT, and 4tOP) did not elicit complete dose-response curves and in some cases behaved as partial agonists on ZELH-zfERα and ZELH-zfERβ2, whereas they had no detectable effect on ZELH-zfERβ1 at test concentrations up to 10 μM (Figs. 5A–7A). In ZELH-zfERα, 4tOP was the most estrogenic compounds, followed by BPA and o,p’-DDT, all three eliciting up to 70% of maximal luciferase activity.

**TABLE 2** EC50 and REP of Various ER Ligands to Induce Luciferase in ZELH-zfERα, ZELH-zfERβ1, and ZELH-zfERβ2 Cell Lines

<table>
<thead>
<tr>
<th></th>
<th>ZELH-zfERα</th>
<th>ZELH-zfERβ1</th>
<th>ZELH-zfERβ2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EC50 (nM)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2</td>
<td>0.20</td>
<td>0.03</td>
<td>0.05</td>
</tr>
<tr>
<td>E1</td>
<td>1.80</td>
<td>0.48</td>
<td>0.48</td>
</tr>
<tr>
<td>E3</td>
<td>2.04</td>
<td>2.00</td>
<td>1.24</td>
</tr>
<tr>
<td>EE2</td>
<td>0.13</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>HEX</td>
<td>0.87</td>
<td>1.62</td>
<td>0.89</td>
</tr>
<tr>
<td>DES</td>
<td>0.06</td>
<td>0.09</td>
<td>0.06</td>
</tr>
<tr>
<td>α-Zea</td>
<td>68</td>
<td>127</td>
<td>265</td>
</tr>
<tr>
<td>β-Zee</td>
<td>144</td>
<td>154</td>
<td>1893</td>
</tr>
<tr>
<td>Gen</td>
<td>277</td>
<td>553</td>
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<tr>
<td>BP1</td>
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<td>3859</td>
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<tr>
<td>BP2</td>
<td>1084</td>
<td>2216</td>
<td>1477</td>
</tr>
<tr>
<td>BP3</td>
<td>n.e.</td>
<td>n.e.</td>
<td>w.e.</td>
</tr>
<tr>
<td>THB</td>
<td>1062</td>
<td>3718</td>
<td>3902</td>
</tr>
<tr>
<td>BPA</td>
<td>1167</td>
<td>3718</td>
<td>3902</td>
</tr>
<tr>
<td>4tOP</td>
<td>332</td>
<td>n.e.</td>
<td>242</td>
</tr>
<tr>
<td>o,p’-DDT</td>
<td>1359</td>
<td>2106</td>
<td>217</td>
</tr>
</tbody>
</table>

*Note. n = number of independent experiments; n.e. = no effect; w.e. = chemicals that induced very weak estrogenic effect (i.e., by less than 20% over solvent control).*
signal. In ZELH-zfERβ2, o,p'-DDT and 4tOP were 10-fold more estrogenic than BPA. Interestingly, o,p'-DDT was fivefold more estrogenic in ZELH-zfERβ2 than in ZELH-zfERα.

Some benzophenone derivatives (BPs) used as ultraviolet (UV) screens and well known as ER ligands were also tested in this study (Figs. 5D–7D). Based on their EC50 (Table 2), BPs exerted almost the same pattern of response in the three cell lines, with a similar ranking: BP2 > THB > BP1, and slightly higher affinity for the zfERα than for the beta subtypes. BP3 had only a very weak effect on ZELH-zfERβ2 cells but not effect in the two other cell lines nor on the ZELH cells (data not shown).

FIG. 5. Dose response curves of luciferase induction in ZELH-zfERα by various ER ligands: (A) natural estrogens: 17β-estradiol (E2), estrone (E1), and estriol (E3); industrial chemicals: 4-tert-octylphenol (4tOP), bisphenol A (BPA), and 1,1,1-trichloro-2-(2-chlorophenyl)-2-(4-chlorophenyl)ethane (o,p'-DDT) (B) pharmaceutical compounds: diethylstilbestrol (DES), hexestrol (Hex), and 17α-ethynylestradiol (EE2), (C) α-zearalenol (α-Zea), β-zearalenol (β-Zee), α-zearalanol (α-Zea), and genistein (Gen), (D) benzophenone derivate: 2,4-dihydroxybenzophenone (benzophenone 1 or BP1), 2,2',4,4'-tetrahydroxybenzophenone (benzophenone 2 or BP2), and 2,4,4'-trihydroxybenzophenone (THB). Cells were exposed for 72 h; values are expressed as percentage of luciferase induction by E2 10nM (means ± SD of triplicates).

**DISCUSSION**

The main outcome of this study was the establishment of a new zebrafish specific *in vitro* reporter gene assay based on the expression of functional zfER subtypes in a zebrafish liver cellular context. We showed that such assay is useful for assessing the estrogenic potency of compounds toward the three different zfER subtypes, hence allowing detection of selective zfER activation by chemicals.

One main advantage is the expression of the three zfER subtypes within the same ZELH clone, allowing direct
comparison of receptor functioning in zebrafish cell. The stability of the system allows reproducible measurement in a sensitive and more convenient way than using transient transfection. Moreover, the very good correlation observed with transient transfection confirmed that the responsiveness of the stable assay to estrogens is representative of this cell model (Fig. 1, Table 1). Overall, the developed cell lines responded to reference estrogens in a similar manner (i.e., same range of active concentrations) than other in vitro assays previously reported using zfERs, which helped validate our experimental system. The natural (E2, E2, and E3) and synthetic estrogens (EE2) induced complete transcriptional activity in the three models, with higher affinity of zfERβ subtypes, which is in line with previous studies using transient transfection of zfERs in human U251-MG and HEK293 cells (Legler et al., 2002; Le Page et al., 2006). Furthermore, the zebrafish cell assay was found more sensitive than human cell–based assays to E2 and to other estrogens, especially when assessing effect on zfERβs. However, our results slightly contrast with those obtained in transiently transfected HeLa cells, in which E2 was slightly more potent on zfERα than zfERβs (Sassi-Messai et al., 2009). EE2 was more active than E2 in each model, as has been

FIG. 6. Dose response curves of luciferase induction in ZELH-zfERβ1 by various ER ligands: (A) natural estrogens: 17β-estradiol (E2), estrone (E1), and estriol (E3), (B) pharmaceutical compounds: diethylstilbestrol (DES), hexestrol (Hex), and 17α-ethynylestradiol (EE2), (C) α-zearalenol (α-Zee), β-zearalenol (β-Zee),α-zearalanol (α-Zea), and genistein (Gen), (D) benzophenone derivates: 2,4-dihydroxybenzophenone (benzophenone 1 or BP1), 2,2′,4,4′-tetrahydroxybenzophenone (benzophenone 2 or BP2), and 2,4,4′-tri hydroxybenzophenone (THB). Cells were exposed for 72 h; values are expressed as percentage of luciferase induction by E2 3nM (means ± SD of triplicates).
already reported in other in vitro models based on fish ER (Ackermann et al., 2002; Cosnefroy et al., 2009; Le Page et al., 2006). Also, the weaker transcriptional activity of zfER\(\beta\)1 compared with that of the two other zfER isoforms in zebrafish cells is consistent with previous reports in other cell lines (Bardet et al., 2002; Le Page et al., 2006; Menuet et al., 2004; Notch and Mayer, 2011).

Some differences however came from the level of luciferase induction that differed from one study or one cell type to another. In our study, the maximal luciferase induction factor was quite low as compared with that described with transient reporter gene assays. For instance, in Chinese hamster ovary (CHO) cells, by using the same expression vectors for zfERs as in the present study, luciferase induction factors by E2 were 7, 9, and 11 times over the control level for ZELH-zfER\(\alpha\), ZELH-zfER\(\beta\)1, and ZELH-zfER\(\beta\)2, respectively (Menuet et al., 2004), whereas in human U251-MG cells, it reached 70-, 10-, and 30-fold, respectively (Le Page et al., 2006).

In U251-MG cells, however, the very high responsiveness was found to be related to the use of the estrogen-regulated

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**FIG. 7.** Dose response curves of luciferase induction in ZELH-zfER\(\beta\)2 by various ER ligands: (A) natural estrogens: 17\(\beta\)-estradiol (E2), estrone (E1), and estriol (E3); industrial chemicals: 4-tert-octylphenol (4tOP), bisphenol A (BPA), and 1,1,1-trichloro-2-(2-chlorophenyl)-2-(4-chlorophenyl)ethane (\(o,p'\)-DDT) (B) pharmaceutical compounds: diethylstilbestrol (DES), hexestrol (Hex), and 17\(\alpha\)-ethynylestradiol (EE2), (C) \(\alpha\)-zearalenol (\(\alpha\)-Zea), \(\beta\)-zearalenol (\(\beta\)-Zee), \(\alpha\)-zearalanol (\(\alpha\)-Zea), and genistein (Gen), (D) benzophenone derivate: 2,4-dihydroxybenzophenone (benzophenone 1 or BP1), 2,2',4,4'-tetrahydroxybenzophenone (benzophenone 2 or BP2), and 2,4,4'-tri hydroxybenzophenone (THB). Cells were exposed for 72 h; values are expressed as percentage of luciferase induction by E2 3nM (means ± SD of triplicates).
zebrafish cyp19a1b promoter within the specific glial cell context, which expresses specific glial transcription factor that potentiates estrogenic activation of this promoter (Le Page et al., 2008). In HeLa cells, induction factors were in the same range as in the present study, i.e., 2.5-fold for zfERα and fivefold for zfERβs (Sassi-Messai et al., 2009). These differences could be explained in part by the different cellular contexts. Indeed, mechanisms of ER-mediated gene regulation are complex and imply recruitment of specific factors that may be cell specific and thus differently affect regulated signaling pathways according to cell context (Klinge, 2001). It is thus likely that the observed response in ZFL cells is relevant to this specific cell context, i.e., zebrafish hepatocyte derived cells.

E2-Activated zfERα and zfERβ2 Inhibited Cell Proliferation

In order to further characterize the newly established zebrafish models, an attempt was made to assess the effect of E2 on endogenous cell responses, including cell proliferation and ER-regulated genes. Impact on proliferation can be used to study estrogenicity of chemicals like E-SCREEN proliferation assay using estrogen-dependent cancer cell lines like MCF-7 or T47D cells (Soto et al., 1997). In addition, it has been reported that E2 has antiproliferative effect in ER-negative cells that have been transfected with ER, such as HeLa (human uterine cervix carcinoma cells) and CHO cells (Garcia et al., 1992; Kushner et al., 1990; Molina-Molina et al., 2008). In such models, inhibition of cell proliferation has been proposed as an estrogenic endpoint to assess activity of chemical on natural endogenous cellular response mediated by the ER (Molina-Molina et al., 2008). Accordingly, in our study, the introduction of the zfER could alter cell growth as we showed significant inhibitory effect of E2 on growth of ZELH-zfERα and ZELH-zfERβ2 cells, whereas cell proliferation of ZELH-zfERβ1 cells was apparently not affected. These observations suggested that zfERα and zfERβ2 were able to interfere with endogenous signaling pathways and thereby to alter the regulation of genes involved in cell proliferation of ZFL. The underlying mechanisms remain, however, to be elucidated. To further explore recombinant zfER interference with endogenous cellular response, an attempt was made by targeting vtg1 and vtg2 gene expression, two hepatic genes regulated by ER in vivo. However, these preliminary experiments failed to detect any upregulation after E2 exposure (data not shown). Further experiments, for instance, using functional genomics, would be helpful to further characterize the established cell lines as potential in vitro tools to identify target genes of zfERs in zebrafish hepatic cell context.

The lack of effect of E2 on the proliferation of ZELH-zfERβ1 cell suggests that zfERβ1 may not be able to interfere with the cellular transcriptional machinery responsible for cell proliferation in this cell line. This can be related to the much lower mRNA expression level of this receptor as compared with the two other zfER isoforms in recombinant cell lines (Fig. 3), although it was found enough functional to elicit activation of the luciferase reporter gene in ZELH-zfERβ1 cells. Another explanation may be related to different physiological roles and functions between zfER isoforms. In vivo, in adult zebrafish liver, E2 was found to downregulate zfERβ1, whereas it strongly upregulated zfERα (Muenet et al., 2004). It is thus likely that the studied hepatic ZFL cell context may not represent a favorable context for its optimal expression and transcriptional activity.

Selective Activation of zfERs by Chemical Ligands

The chemical screening showed that ER ligands from different chemical classes were able to induce transactivation of zebrafish ERs. It also highlighted differences on their estrogenic potencies depending on examined zfER subtypes, hence suggesting some zfER selectivity for certain ligands. This selectivity also differs from that observed between human ERα and ERβ orthologs. Although zfERα and zfERβs belong to ERα and ERβ phylogenetic groups, they share only ~40–50% identity with human ER subtypes based on amino acid sequences, respectively (Muenet et al., 2002). Such differences likely contribute to different sensitivity or selectivity of ER to chemicals across species.

In our system, zfERβ2 presented higher affinity than zfERα for estrogens E2, E1, and EE2 (Table 2), unlike what has been described on human ERs subtypes affinity for these substances (Escande et al., 2006). Interestingly, higher sensitivity to E2 of ERβ2 subtypes has also been reported for medaka, despite dissimilarities between zebrafish and medaka ER sequences (Chakraborty et al., 2011). Similarly, the well-described human ER ligand o,p′-DDT also presented marked preferential activity on zfERβ2 than zfERα. BPA was recently shown to activate both human ER subtypes at same concentrations (Riu et al., 2011), as we found here using zebrafish receptors (Table 2). Another marked difference between zebrafish and human concerns genistein, a selective human ERβ modulator (Barkhem et al., 1998; Escande et al., 2006), which had slightly higher affinity for zfERα than for zfERβ subtypes. Our results are in line with those of Sassi-Messai et al. (2009) using transient transfection of zfERs in HeLa cells. Conversely, Le Page et al. (2006) described zfERβ2, but not zfERβ1, as having higher affinity for genistein than zfERα. It is noted that Gen was the only compound tested that led to nonspecific increase of luciferase in ZELH cells, which do not express functional ER, with an overexpression of luciferase by 20% over control at 10μM (data not shown); however, we assume that this nonspecific response did not impede assessment of its estrogenic effect at lower concentrations.

Other chemicals, including zearalenone metabolites and BPs, activated zfERα at lower concentrations than zfERβs (Table 2). Zearalenone metabolites have been well described as ER agonists in both fish and human in vitro systems (Le Guevel and Pakdel, 2001). Moreover, zearalenone and α-Zea have been shown to bind similarly to human ERα and ERβ.
In our study, zearalenone metabolites generally behave as partial agonists for the three receptors, except β-Zee in ZELH-zfERβ1 cells, and were active on zfERα at slightly lower concentration than on zfERβs. Such partial activity of zearalenone metabolites was in line with previous observation in other reporter gene cell assays using the rainbow trout (Cosnefroy et al., 2009) and human ERs (Pillon et al., 2005). According to our results, this mycoestrogen was found as the less estrogenic among different zearalenone metabolites on hERα in vitro (Le Guevel and Pakdel, 2001).

BPs are mostly used in personal care products as UV filters. These lipophilic chemicals have been detected in environment, in water, and in fish (Balmer et al., 2005), and several BPs have been well described as ligands for human and fish ERs (Kunz et al., 2006; Molina-Molina et al., 2008; Suzuki et al., 2005). In the present study, BP1, BP2, and THB exerted estrogenic potency on the three ZELH-zfERs cell models, with slightly higher activity on zfERβ3. BP3 induced a weak estrogenic activity on ZELH-zfERβ2 and was inactive in the two others models. Based on their EC50, similar potency and ranking of these four BPs were previously reported in different in vitro models such as HeLa cells expressing hERα, hERβ, or rτERα (Molina-Molina et al., 2008) or in the Poeciliopsis lucida hepatocellular carcinoma line 1 cells expressing the rτERα (Cosnefroy et al., 2009). However, a different ranking was observed in yeast cells expressing the rτERα (Kunz et al., 2006), which may rather come from differences in chemical uptake and bioavailability and cell metabolic capacity of the assay than receptor affinity and functioning (Fang et al., 2000). Another remarkable result is the finding that BPs were more active on zfERα, which contrasts with their higher activity on hERβ than on the hERα reported in HeLa cells (Molina-Molina et al., 2008). To our knowledge, BPs have not so far been tested on different subtypes of zebrafish ERs, as presented in our study. Such difference, however, reinforces the observation of differential selective ER binding affinity and activation between receptor subtypes when comparing human and zebrafish ERs, as discussed above.

CONCLUSIONS

In this study, we successfully developed a zebrafish specific in vitro stable reporter gene assay, which allows assessment of the estrogenic potency of chemicals toward the three zfER subtypes, namely zfERα, zfERβ1, and zfERβ2, in the ZFL cell line. Such assay serves as a screening tool to characterize estrogenic activity of chemicals toward zebrafish ERs and to identify possible selective activators of zebrafish ER subtype. Our data notably showed the relevance this zebrafish specific in vitro assay to highlight species differences in estrogenic potencies, as compared with in vitro data using human ERs. In addition, this study stresses the need to further increase knowledge on the role of zfER isoforms in teleost.

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REFERENCES


