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Selectivity of natural, synthetic and environmental estrogens for zebrafish estrogen receptors

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ABSTRACT

Zebrafish, *Danio rerio*, is increasingly used as an animal model to study the effects of pharmaceuticals and environmental estrogens. As most of these estrogens have only been tested on human estrogen receptors (ERs), it is necessary to measure their effects on zebrafish ERs. In humans there are two distinct nuclear ERs (hERα and hERβ), whereas the zebrafish genome encodes three ERs, zfERα and two zfERβs (zfERβ1 and zfERβ2). In this study, we established HeLa-based reporter cell lines stably expressing each of the three zfERs. We first reported that estrogens more efficiently activate the zfERs at 28 °C as compared to 37 °C, thus reflecting the physiological temperature of zebrafish in wildlife. We then showed significant differences in the ability of agonist and antagonist estrogens to modulate activation of the three zfER isotypes in comparison to hERs. Environmental compounds (bisphenol A, alkylphenols, mycoestrogens) which are hER panagonists and hERβ selective agonists displayed greater potency for zfERα as compared to zfERβs. Among hERα selective synthetic agonists, PPT did not activate zfERα while 16α-LE2 was the most zfERα selective compound. Altogether, these results confirm that all hER ligands control in a similar manner the transcriptional activity of zfERs although significant differences in selectivity were observed among subtypes. The zfER subtype selective ligands that we identified thus represent new valuable tools to dissect the physiological roles of the different zfERs. Finally, our work also points out that care has to be taken in transposing the results obtained using the zebrafish as a model for human physiopathology.

Introduction

Estrogens play critical roles in various physiological processes during the development and adult life of vertebrates. In mammals, these effects are mediated by members of the nuclear receptor superfamily, the estrogen receptors ERα and ERβ (Green et al., 1986; Kuiper et al., 1996). These receptors share a common structural architecture composed of three functional domains. The A/B (or NH2-terminal domain) is involved in transcriptional activation of gene expression; the C or DNA-binding domain contains a two zinc finger structure, which is important for receptor dimerization and binding of receptors to specific DNA sequences; the E/F or COOH-terminal domain mediates ligand binding, receptor dimerization, nuclear translocation and transactivation of target gene expression in association with coactivators and corepressors (Nilsson et al., 2001). The transactivation functions of both the A/B (AF-1) and the E/F (AF-2) domains are dependent on the cell type and promoter context (Berry et al., 1990). Some ER subtype-selective

Abbreviations: hERs, human estrogen receptors; zfERs, zebrafish estrogen receptors; AF, transactivation function; LBD, ligand binding domain; EDCs, endocrine disrupting chemicals; E2, 17β-estradiol; 17α-E2, 17α-estradiol; E1, estrone; E3, estriol; EE2, 17α-ethynylestradiol; BP2, benzophenone-2; PPT, 4,4′,4″-(4-propyl-[^{1}H]-pyrazole-1,3,5-triyl)trisphenol; DPN, 2,3-bis(4-hydroxyphenyl)-propionitrile; WAY200070, (7-bromo-2-(4-hydroxyphenyl)-1,3-benzoxazol-5-ol); FERB033, (2-chloro-3′-fluoro-3,4′-dihydroxy-[1,1-biphenyl]-4-carboxaldehyde oxime); ERB041, (7-ethenyl-2-(3-fluoro-4-hydroxyphenyl)-5-benzoxazolol); MPP dihydrochloride, (1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1*H*-pyrazole dihydrochloride); PHTPP, (1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1*H*-pyrazole dihydrochloride); 4OH-Tam, 4-hydroxytamoxifen; ICI 182,780, (7α,17β-[9[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol); 16α-LE2, 3,17-dihydroxy-19-nor-17α-pregna-1,3,5(10)-triene-21,16α-lactone; 8β-VE2, 8-vinylestra-1,3,5(10)-triene-3,17β-diol.

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ligands have been identified, which have different binding affinities for the two estrogen receptors and present variable agonistic or antagonistic characters depending on the ER considered (Delfosse et al., 2012; Escande et al., 2006; Molina-Molina et al., 2008). Dissimilarities in the N-terminal and ligand binding domain (LBD) regions of ERα and ERβ explain the differences between the two receptors in their response to various ligands (Matthews and Gustafsson, 2003; Ogawa et al., 1998).

Even though in mammals only two ER subtypes have been characterized, the presence of three ER subtypes has been reported in teleosts including the zebrafish (*Danio rerio*) (Hawkins et al., 2000; Ma et al., 2000; Menuet et al., 2002). Zebrafish ERα (esr1) is orthologous to the human ERα, while ERβ1 (esr2b) and ERβ2 (esr2a) are orthologs of the human ERβ (Bardet et al., 2002). The overall amino-acid sequence identity between the zfER subtypes and their corresponding human ER orthologs is approximately 50% (Menuet et al., 2002). Since these three zfERs are thought to mediate different biological effects, there is an increased interest in finding subtype-selective zfER ligands.

Xenoestrogens represent a class of endocrine disruptors that affect estrogen signaling. This class of compounds is composed of natural estrogens (phyto- and mycoestrogens), and synthetically derived agents including certain pharmaceuticals, pesticides and industrial compounds used in consumer goods (Singleton and Khan, 2003). Zebrafish is a worldwide recognized vertebrate model to investigate the mode of action of endocrine disrupting chemicals (EDCs) and their reproductive and developmental effects (Segner, 2009). Studies on zebrafish have led to significant advances on the effect of EDCs on ER-regulated pathways through the use of zebrafish-specific in vitro and in vivo models (Menuet et al., 2002; Brion et al., 2012; Cosnefroy et al., 2012; Gorelick et al., 2014). Although all three zfERs subtypes can be activated by 17β-estradiol (E2), similarly to the mammalian ERs, it has been shown that the capacity of some compounds to transcriptionally activate the estrogen receptors differs between zebrafish and mammals. For instance, the phytoestrogen genistein and the benzophenones BP1, THB and BP2 which are selective human ERβ agonists (Escande et al., 2006; Molina-Molina et al., 2008) preferentially activate zfERα (Cosnefroy et al., 2012). However, a detailed analysis of similarities and differences in ligand specificity between zfERs and hERs remains to be performed. The evaluation of the activity and transcriptional profiles of known mammalian estrogenic ligands in the zebrafish model can provide additional information for the analysis of ER-mediated processes in this organism including disruption of these processes by xenoestrogens (Notch and Mayer., 2011).

The purpose of this study was to evaluate the effects of several known natural, environmental and pharmaceutical (anti)estrogenic compounds on the transcriptional activity of the three zfERs and to compare the data with their activity on hERs. To address this challenge, zfERα, zfERβ1 and zfERβ2 reporter cell lines were established in the same cellular context as we previously used to create hERα and hERβ cell lines (Balaguer et al., 1999). In HeLa cells, which stably express an ERE-driven luciferase reporter (HELN cells), we expressed the full-length zfERα, zfERβ1 and zfERβ2, respectively. The resulting HELN-zfER cell lines are useful tools for the analysis of the effects of estrogenic compounds on gene transactivation by the three zfERs, and also for the comparison of these effects to the results obtained on the hER orthologs. Since zebrafish is used as a model for the study of the effects of xenoestrogens in vivo, determining the transcriptional profiles of estrogenic compounds on the zfERs is crucial to support the zebrafish model for ER-related studies and their extrapolation to the mammalian system.

Materials and methods

Materials. Tissue culture plates used in this study came from Greiner Bio-one (Monroe, NC, USA), and media was purchased from Invitrogen (Grand Island, NY, USA). Luciferin (sodium salt) was purchased from Promega (Charbonnières, France). 17β-Estradiol (E2) (1,3,5[10]-estratriene-3,17β-diol), 17α-estradiol (17α-E2) (1,3,5[10]-estratriene-3,17α-diol), estrone (E1) (1,3,5[10]-estratriene-3-ol-17-one), estriol (E3) (1,3,5[10]-estratriene-3,16α,17β-triol), 17α-ethynylestradiol (EE2) (17α-ethynyl-1,3,5[10]-estratriene-3,17β-diol), genistein (40,5,7-trihydroxyisoflavone), benzophenone-2 (BP2) (2,2′,4,4′-tetrahydroxybenzophenone), α-zearalanol, α-zearalenol, 4-tert-octylphenol and bisphenol A were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). 4,4′,4″-(4-Propyl-[^{1}H]-pyrazole-1,3,5-triyl)trisphenol (PPT), 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN), WAY200070 (7-bromo-2-(4-hydroxyphenyl)-1,3-benzoxazol-5-ol), FERB033 (2-chloro-3′-fluoro-3,4′-dihydroxy-[1,1-biphenyl]-4-carboxaldehyde oxime), ERB041 (7-ethenyl-2-(3-fluoro-4-hydroxyphenyl)-5-benzoxazolol), MPP dihydrochloride (1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1*H*-pyrazole dihydrochloride), PHTPP (1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1*H*-pyrazole dihydrochloride) and liquiritigenin were purchased from Tocris Bioscience (Minneapolis, MN, USA). Ferutinin was purchased from Santa Cruz Biotechnology Inc. (Dallas, Texas, USA). 4-Hydroxytamoxifen (4OH-Tam) (1-[p-dimethylaminoethoxyphenyl]-1-(4-hydroxyphenyl)-2-phenyl-1-butene) and ICI 182,780 (7α,17β-[9[(4,4,5,5,5-pentafluoropentyl) sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol) were obtained from Zeneca (Macclesfield, UK). Raloxifene (6-hydroxy-3-[4-[2-(1-piperidinyl)ethoxy]phenoxy]-2-(4-hydroxy phenyl)-benzothiophene) came from Eli Lilly (Indianapolis, IN, USA). 3,17-Dihydroxy-19-nor-17α-pregna-1,3,5(10)-triene-21,16α-lactone, named 16α-LE2 and 8-vinylestra-1,3,5(10)-triene-3,17β-diol, named 8β-VE2 is a kind gift of Peter Muhn from Research Laboratories of Schering AG (Berlin, Germany). Compounds were dissolved in dimethyl sulfoxide (DMSO) as 10^{-2} M stock solutions and successive dilutions were performed in cell culture medium. The final DMSO concentration never exceeded 0.1% (v/v) of the culture medium.

Cell lines. HELN-zfERα, -zfERβ1 and -zfERβ2 reporter cell lines were established in a similar way as HELN-hERα and -hERβ cell lines (Balaguer et al., 1999). Briefly, HELN-zfERα, β1 and β2 cell lines cells were obtained by transfection of HELN cells (HeLa cells stably transfected with the ERE-βGlobin-Luc-SVNeo plasmid) (Balaguer et al., 1999) by the corresponding pSG5-puro plasmids (pSG5-zfERα-puro, pSG5-zfERβ1-puro and pSG5-zfERβ2-puro, respectively). Cells were selected by Geneticin (G418, Sigma-Aldrich) and puromycin (Sigma-Aldrich) at the final concentration of 1 mg/mL and 0.5 μg/mL, respectively. The best responsive clones were selected based on both EC50 values and luciferase induction factors of E2.

Cell culture conditions. The HELN zfERs and hERs cell lines were cultured in phenol red-free Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) and supplemented with 5% dextran-coated charcoal-treated fetal bovine serum (FBS-DCC) and 1% antibiotic (penicillin/streptomycin) in a 5% CO_2 humidified atmosphere at 37 °C. Selection agents (Geneticin 1 mg/mL and puromycin 0.25 μg/mL) were maintained in the cell culture medium. Luciferase assays were performed in 5% FBS-DCC medium.

zfERα, zfERβ1 and zfERβ2 transactivation assays. The HELN-zfERs cell lines were seeded at a density of 80,000 cells per well in 96-well white opaque tissue culture plates (Greiner CellStar, USA). Compounds to be tested were added 8 h later, and cells were incubated with compounds at 28 °C for 16 h. Cells were maintained in the presence of ligands at 28 °C because of the improved transactivation ability of the zfERs at this temperature. Experiments were performed in quadruplicates in at least two independent experiments. At the end of the incubation period, culture medium was replaced with medium containing 0.3 mM luciferin. Luciferase activity was measured for 2 s in intact living cells using a plate reader (PerkinElmer Luminometer, MA, USA). EC50