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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 zfERβ (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 zfERs isotypes in comparison to hERs. Environmental compounds (bisphenol A, alkylphenols, mycoestrogens) which are hERs 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 amongst subtypes. The zfER subtype selective ligands that we identified thus represent new valuable tools to dissect the physiological roles of the differents 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.

Keywords: Estrogen receptors, zebrafish, selective estrogens, endocrine disruptors, reporter cell lines

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Abbreviations : hERs, human estrogen receptors; zfERs, zebrafish estrogen receptors; AF, transactivation function ; LBD, ligang 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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Introduction

Estrogens play critical roles in various physiological processes during development and adult life in 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. 1987). 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 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 bindind domain (LBD) regions of ERα and ERβ explain the differences between the two receptors in their response to various ligands (Matthews et al. 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

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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 et al. 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 et al. 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; Gorelik 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-zfERs 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.

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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-3,17 α -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), 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-(1piperidinyl)ethoxy]phenoxy]-2-(4-hydroxy phenyl)-benzothiophene) came from Eli Lilly (Indianapolis, IN, USA). 3,17-

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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 EC_{50} 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)

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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 values were measured using GraphPad Prism software (version 5.04; Graphpad Software Inc., San Diego, CA, USA). hERα and hERβ1 transactivation assays were performed in a similar manner except for the incubation temperature with the ligands (37°C instead of 28°C).

zfERs binding assays

The HELN-zfERs cell lines were seeded at a density of 80,000 cells per well in 96-well white bottom clear tissue culture plates (Greiner CellStar, USA). [^{3}H]-E2 3 nM in the presence or in the absence of non-radioactive E2 10 µM were added 8 h later, and cells were incubated at 28°C or 37°C for 16h. At the end of the incubation period, unbound material was aspirated and cells

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washed three times with PBS. Then, 0.2 ml of LSC-cocktail (Emulsifier-Safe, Packard Bioscience) was added and [^{3}H]-bound radioactivity was counted. Protein concentrations were measured by Bio-Rad protein assay and used to normalize bound radioactivity values expressed in dpm. Experiments were performed in quadruplicate in at least two independent experiments.

Results

Impact of the temperature on transcriptional activation of zfERs.

Since the functionality of rainbow trout ER has been shown to be temperature-sensitive (Cosnefroy et al. 2009 ; Matthews et al. 2002), we first tested the ability of E2 to activate the zfERs at different temperatures, including a temperature relevant to the physiology of this organism. As seen in Fig. 1A, the EC_{50} value of E2 was lower at 28°C (0.077 nM) than at 31°C (0.266 nM), 34°C (0.433 nM) or 37°C (1.921 nM) for zfERα. Similarly, the EE2 EC_{50} value was lower at 28°C (0.061 nM) than at 31°C (0.127 nM), 34°C (0.19 nM) or 37°C (0.947 nM) (Fig. 1B). EC_{50} of zfERβ1 and zfERβ2 cell lines were also sensitive to the température (Fig. 1C and 1D). EC_{50} values for E2 and EE2 were 0.039 and 0.031 nM at 28°C and 0.714 and 0.673 nM at 37°C for zfERβ1. EC_{50} values for E2 and EE2 were approximately 0.118 and 0.031 nM at 28°C and 1.554 and 0.197 nM at 37°C for zfERβ2. As we suspected that the increase in the EC_{50} values was due to zfERs degradation, we measured protein level by saturation ligand binding assay with [^{3}H-E2]. In HELN-zfER cells, zfER concentration was 8-fold lower at 37°C than 28°C (data not shown).

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Effects of human endogenous estrogens on zfERs transcriptional activation

The measured EC50 values for E2 indicates that E2 has a slight zfERβ1 selectivity relative to the other zfER subtypes in this cell context. The reported EC50 values for E2 is approximately 0.017 nM for hERα and 0.068 nM for hERβ (Escande et al. 2006), suggesting that E2 has approximately a 6-fold higher potency to transactivate luciferase gene expression in hERα relative to its zebrafish counterpart. 17α-Ethynilestradiol (EE2), which has a 31-fold greater potency to transactivate the luciferase reporter gene in HELN-hERα compared to hERβ (Escande et al. 2006), demonstrated equal agonistic activities on all 3 zfERs with EC values of 0.061 nM for zfERα and 0.031 nM for both zfERβ isoforms (Table 1).

We next evaluated the effects of the weak estrogenic stereoisomer of 17α-estradiol (17α-E2), estrone (E1) and estriol (E3), on zfER-mediated transcriptional activation (Fig. 2). These endogenous ligands have been shown to behave as full agonists in both of the human ERs with a slight ERα selectivity (Escande et al. 2006 and supplementary data Fig. S1A). The transcriptional profiles obtained by E1, E3 and 17α-E2 indicate that these estrogens have a stronger potency in transactivating zfERα compared to the zfERβ1 and zfERβ2 isoforms (Fig. 2 A, B, C). E1 and E3 displayed similar potencies on zfERα (EC50 values of 1.7 and 0.55 nM, respectively), zfERβ1 (EC50 values of 5.6 and 2.6 nM, respectively) and zfERβ2 (EC50 values of 12.1 and 8.5 nM, respectively). Among the endogenous estrogens tested, 17α-E2 showed the weakest agonistic activity with EC50s of 5.3, 24.7 and 45.1 nM for zfERα, zfERβ1 and zfERβ2, respectively.

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Selectivity of natural estrogens on zfERs transcriptional activity

We next analyzed the ability of myco and phytoestrogens to activate the zfERs (Fig. 3). The mycoestrogen α-zearalanol (α-zea) exhibited EC50 values of 0.176 nM for zfERα, 0.71 nM for zfERβ1 and 1.2 nM for zfERβ2, which conferred 4-fold and 6.8-fold higher potencies in transactivation assays for HELN-zfERα compared to the zfERβ cell lines (Fig. 3A). The other zearalanone analogues (zearalanone, β-zearalanol, zearalenone, α-zearalenol and β-zearalenol) presented a similar profile (zfERα potency > zfERβ1 potency ≥ β2 potency, supplementary data Fig. S2A and data not shown). The phytoestrogen genistein, which present a higher affinity for hERβ than for hERα (Escande et al. 2006; Molina-Molina et al. 2008), demonstrated greater affinity for zfERα and zfERβ2 relative to zfERβ1 (Fig. 3B). Genistein activated zfERα and β2 with a 3.9-4.4 greater potency than zfERβ1 with EC50 values of 40, 50 and 260 nM for zfERα, zfERβ2 and zfERβ1, respectively. Liquiritigenin, another phytoestrogen with higher affinity for hERβ than for hERα (supplementary data Fig. S1D), activated zfERα with a 12 greater potency than zfERβ2 with EC50 values of 563 and 6805 nM for zfERα and zfERβ2, respectively, and did not activate zfERβ1 at all (table 1 and Fig. 3C). Finally, ferutinin, a phytoestrogen with similar affinity for hERα and hERβ but with different maximal activity (Table 1 and supplementary data Fig. S1E) displayed similar maximal activity (100%) but different potency for the zfERs (EC50 values of 8, 3.4 and 132 nM for zfERα, zfERβ1 and zfERβ2, respectively) (Table 1 and Fig 3.D).

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Selectivity of environmental compounds on zfERs transcriptional activity

We next analyzed the ability of known environmental estrogenic compounds to activate the zfERs (Fig. 4). Similarly to the human ERs (Delfosse et al. 2012), bisphenol A (BPA) acted as a partial agonist towards the 3 zfERs (Fig. 4A). BPA displayed greater affinity for zfERα than the zfERβ subtypes. The EC50 values of BPA were 599 nM, 1.894 µM and 3.823 µM for zfERα, zfERβ1 and zfERβ2, respectively (Table 1). The alkylphenol 4-tert-octylphenol (4t-OP) and the nonylphenol mixture (NPm) acted as partial agonists towards zfERα and zfERβ2 and full agonists towards zfERβ1 (supplementary data Fig. 4B and 4C). 4tOP and NPm displayed greater affinity towards zfERα and zfERβ2 relative to zfERβ1. The EC50 values of 4tOP and NPm were 763 and 487 nM for zfERα, 2478 and 4225 nM for zfERβ1 and 1223 and 603 nM for zfERβ2. Benzophenone 2 (BP2), which present a higher affinity for hERβ than for hERα (Molina-Molina et al. 2008), demonstrated greater affinity for zfERα and zfERβ2 relative to zfERβ1 (Fig. 4D). BP2 presented a 3-fold higher potency towards zfERα and zfERβ2 compared to zfERβ1 (EC50 values of approximately 251 nM and 233 for zfERα and β2 respectively, and 803 nM for β1).

Selectivity of of pharmaceuticals on zfERs transcriptional activity

We next evaluated the ability of synthetic ligands to activate transcription through the zfERs. The human ERα selective compound 3,17-dihydroxy-19-nor-17α-pregna-1,3,5(10)-triene-21,16α-lactone (16α-LE2), which has over 1000-fold selectivity in reporter gene activation through hERα than hERβ (Escande et al. 2006) also showed greater selectivity for its zebrafish ERα counterpart relative to the zfERβ isoforms (Fig. 4A). Among all the compounds tested, 16α-LE2 displayed the greatest zfERα selectivity with approximately 429 and 115-fold higher potencies to activate

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transcription by zfERα compared to zfERβ1 and zfERβ2, respectively. The selective ERα selective compound 4,4',4''-(4-Propyl-[1*H*]-pyrazole-1,3,5--triyl)*tris*phenol (PPT) did not activate zfERα at all while it partially activated zfERβ1 and zfERβ2 (Fig. 4B). Even though 8-vinylestra-1,3,5(10)-triene-3,17β-diol (8βVE2) and 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN) are human ERβ selective agonists, these compounds showed better selectivity for zfERα than for the zfERβ isoforms (Fig. 4C and 4D). The EC50 values for 8βVE2 were 32, 188 and 113 nM for zfERα, zfERβ1 and zfERβ2, respectively (Table 1). Likewise, the EC50 values of DPN were 21.7, 238 and 115 nM for zfERα, zfERβ1 and zfERβ2, respectively (Table 1). Similarly, other human ERβ selective agonists such as 7-bromo-2-(4-hydroxyphenyl)-1,3-ben-zoxazol-5-ol (WAY300070), 2-chloro-3'-fluoro-3,4'-dihydroxy-[1,-1-biphenyl]-4-carboxaldehyde oxime (FERB33) and 7-ethenyl-2-(3-fluoro-4-hydroxyphen-yl)-5-benzoxazolol (ERB041) displayed greater potencies for zfERα compared to zfERβ1 and zfERβ2 (Table 1 and supplementary data Fig. S2B-D).

Effect of pharmaceutical antagonists on transcriptional activity

The pharmaceutical anti-estrogenic compounds ICI 182,780, raloxifene, 4OH-tamoxifen (4OH-Tam) were tested in the HELN-zfERs and all displayed full antagonistic activity in the three zfER cell lines (Fig. 5A, B, C). No agonistic activity for these three anti-estrogens was observed in our experimental system (data not shown). ICI 182,780 showed slight preferential antagonistic activity towards zfERα (IC50 value of 3 nM) than the zfERβ cell lines (IC50 values of 14.7 nM and 28 nM for zfERβ1 and β2, respectively) (Fig. 5A). 4OH-Tam antagonistic potency was greater for zfERα and zfERβ1 (IC50 values of 8.2 and 4.5nM for zfERα and zfERβ1,

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respectively) than for zfERβ2 (IC50 value of 29.4 nM) (Fig. 5B). Raloxifene which has slight preferential antagonistic activity towards hERα (IC50 value of 0.62 nM) than the hERβ (IC50 value of 12 nM) was a more powerful antagonist for zfERβ1, with a 4.1 and 9.7-fold greater potencies towards the zERβ1 subtype compared to zfERα or zfERβ2, respectively (IC50 values of 57, 13.8 and 134 nM for zfERα, zfERβ1 and zfERβ2, respectively) (Fig. 5C).

Another compound tested for its effect on transcriptional inhibition on the HELN-zfERs was the hERα selective agonist and hERβ antagonist PPT. As shown in figure 5D, PPT displayed full antagonistic activity towards zfERα (IC50 value of 261 nM) and it was able to partially antagonize estrogenic activation in the zfERβ cell lines with IC50 values of 854 and 326 nM for zfERβ1 and zfERβ2, respectively. Finally, antagonists with hERα (MPP) and hERβ (PHTPP) selectivity were tested on the HELN-zfERs reporter cells. MPP presented slight zfERβ1 selectivity and PHTPP behaved as zfERs pan-antagonist (Table 1 and supplementary data Fig. S2E-F).

Structural analysis of zfERα selectivity

To gain structural insights into the zfERα selectivity of 16α-LE_2 in human and zebrafish ERs, we used the web-based server EDMon (Endocrine Disruptor Monitoring; http://atome2.cbs.cnrs.fr/AT2B/SERVER/ EDMon.html) (Delfosse et al. 2012) to model hERα and hERβ in complex with this ligand. The structural basis of the hERα and hERβ selectivity towards certain ligands has been associated with two amino acid differences in their ligand binding pockets. Indeed, L384 and M421 of hERα are replaced by M336 and I373 in hERβ, respectively (Fig. 7 and Manas et al., 2004). Superimposition of the 16α-LE_2-bound hERα model

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on the crystal structure of hERα in complex with E_2 (PDB code 3UUD) showed that the phenol ring of 16α-LE_2 occupies the same position as that of E_2 and is engaged in a network of hydrogen bonds with E353 from helix 3 (H3) and R394 from H5 (Fig. 8A). On the other side of the ligand-binding pocket (LBP), it appears that the hydrogen bond observed between the 17-hydroxyl group of E_2 and H524 (H11) is conserved in 16α-LE_2. The difference between the two complexes resides in the lactone ring of 16α-LE_2 which points towards M421 (H7) that must undergo a large conformational change to accommodate this additional group. In hERβ, the linear M421 is replaced by the branched residue Ileu 373 characterized by a much smaller intrinsic flexibility (Fig. 8B). As a consequence, I373 maintains the synthetic ligand in a position where it interacts unfavorably with M336 (H3). Therefore, 16α-LE_2 adopts different positions in hERα and hERβ, the more constrained environment provided by the latter accounting for the weaker affinity of the ligand for this receptor subtype. The affinity values measured with the zebrafish receptors reflect the variations in the space constraints provided by the different combinations of residues in the three receptor subtypes. With H3 and H7 residues identical to those of the human receptor, zfERα interacts with 16α-LE_2 with the highest affinity. The slight difference in the binding affinity of 16α-LE_2 for hERα and zfERα relies most likely on the replacement of L349 (H3) by a methionine residue (M317) (Fig. 7) and a possible loss of a favorable interaction provided by the branched but not by the linear residue (Fig. 8C). With a conserved isoleucine in H7 (I406) and a leucine residue in H3 (L369) (Fig. 7), zfERβ1 displays the most constrained LBP reflecting the weakest binding affinity for 16α-LE_2. This receptor combines two large residues with low (isoleucine) and medium (leucine) flexibilities (Figure 8D). The replacement of I406 in H7 of zfERβ1 by a leucine residue (L391) (Fig. 7) in zfERβ2 provides a slight gain in LBP plasticity (Figure 8D), in agreement with the slightly better affinity of 16α-LE_2 for the latter.

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The inability of hERβ-selective phytoestrogens (genistein and liquiritigenin) and pharmaceuticals (8bv-E2, DPN, WAY300070, FERB033 and ERB041) to activate the zfERβ isoforms is explained by the mutation of a critical amino acid involved in genistein binding in hERβ. In all zfERs, the position homologous to hERβ M336 is occupied, as in hERα, by a leucine residue (Fig. 7)(Sassi-Messai et al. 2009). This anino acid change most likely accounts for the lack of obvious selectivity of the phytoestrogens towards the zfERβ subtypes.

Discussion

Screening of endogenous, environmental and synthetic ligands in the HELN-zfER cell lines showed that known mammalian ER ligands are also able to induce transcriptional activity of zebrafish ER subtypes. This study allowed us to assess differences in the potency of the estrogenic compounds among the three zfER subtypes, and compare their selectivity towards hERs using a similar human cellular context. The HELN-zfERs cells were incubated at 28°C after addition of chemicals to the cells because it is a more physiologically relevant temperature for zebrafish, which increased the potency of estradiol approximately 10-fold compared to incubation at 37°C (Fig. 1). Temperature sensitivity of fish ERs has already been reported using reporter gene assays (Cosnefroy et al. 2009; Matthews et al. 2002) and the reason seems to be thermo-dependence of estrogen binding (Matthews et al. 2002; Sumida et al. 2003; Tan et al. 1999). Interestingly, this thermo-dependence is not shared by other zebrafish nuclear receptors like the peroxisome proliferator activated receptor γ (Riu et al. 2014).

Results from this study demonstrated that the selectivity of some of the tested compounds on zfERs differs from that observed between hERα and hERβ orthologs. zfERα and zfERβs belong

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to the ERα and ERβ phylogenetic groups, respectively; however, one to three amino-acids differ in the ligand binding domain of the zfERs compared to their human ER counterparts (Table 3), which certainly explains the differences in selectivity and sensitivity of estrogenic ligands between zebrafish and human ERs.

Among the natural estrogens tested in the HELN-zfER cell lines, 17β-estradiol (E2) was the compound with the highest potency towards the three zfERs, followed by estriol (E3), estrone (E1) and 17α-E2. Likewise, a study using transiently-transfected HEK293 cells showed that E2 was the most potent endogenous ligand for zfERα with EC50 of 0.177 nM, very similar to the EC50 value of E2 for zfERα in our study (0.077 nM) (Lange et al. 2012). E2 also is the endogenous compound with the highest affinity for the hERs, and it also displays a better affinity for hERα (Escande et al. 2006). The zfERs were less sensitive to E2 than hERs, with approximately 6 and 2-fold lower transactivation potencies for the zfERα and zfERβ subtypes relative to their human orthologs, respectively.

Among the myco and phytoestrogens, α-zearalanol (α-zea) demonstrated the best agonistic activity via the zfERα and zfERβ forms with the ability to almost fully activate the luciferase reporter at relative low EC50 values. α-zea preferentially activated zfERα in the HELN-zfERs, which is in accordance with our previous studies performed in zebrafish liver-derived cell lines (Cosnefroy et al. 2012). The phytoestrogen genistein, a full agonist in both human ERs with 10-fold higher selectivity for hERβ, showed preferential and equal activity towards zfERα and zfERβ2 over zfERβ1. This phytoestrogen also behaved as a full agonist in the zebrafish cell lines, and displayed slightly higher affinity for zfERα and zfERβ2 than for zfERβ1 (Cosnefroy et al. 2012). Similar transactivation properties by genistein were observed in HeLa cells transiently transfected with the zfERs and an estrogen-dependent luciferase reporter (Sassi-Messai et al.

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2009). Liquiritigenin, another phytoestrogen with higher hERβ then hERα affinity, also displayed preferential and equal activity towards zfERα and zfERβ2 over zfERβ1. Finally, ferutinin, a terpenoid with similar affinity for hERα and hERβ but different activity (full agonist on hERα and partial agonist on hERβ) (Ikeda et al. 2002), behaved as a full agonist on the three zfERs and displayed slightly higher affinity for zfERα and zfERβ1 than for zfERβ2.

Among environmental pollutants, bisphenol A (BPA) also presented higher affinity for zfERα relative to the zfERβ subtypes. Accordingly, in the zebrafish-derived cell lines (Cosnefroy et al. 2012), BPA also displayed better potency towards zfERα. Considering reporter gene activation in the human ERs, BPA displays similar agonistic properties between hERα and hERβ, presenting slightly better efficacy towards hERβ (Delfosse et al. 2012). The alkylphenols 4tOP and NPm also showed better potency towards zfERα than the zfERβ subtypes. The benzophenone derivative BP2 behaved as a full agonist in the three zfERs, with similar EC50s for both zfERα and zfERβ2. Cosnefroy et al. (2012) also found that BP2 has a slightly higher activity towards zfERα, with approximately 1.36 and 2 fold- higher potencies compared to zfERβ2 and β1, respectively (Cosnefroy et al. 2012).

17α-EE2 was the pharmaceutical compound that displayed the highest affinity towards the zfERs, with almost equivalent potencies in transactivating the three zfER subtypes. Similarly to hERs, 16α-E2, which has 1000-fold selectivity for hERα (Escande et al. 2006), also exhibited higher affinity for zfERα compared to the zfERβ subtypes (approximately 100 and 370-fold higher potencies than zfERβ2 and zfERβ1, respectively) and it is the most selective compound for zfERα found in this chemical screening. Conversely, PPT, another hERα selective agonist that is unable to activate hERβ (Escande et al. 2006), was devoid of agonistic activity in the HELN-zfERα cells, and instead behaved as an antagonist to zfERα, while it slightly activated the zfERβ

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subtypes. IC50 values obtained in zfERs cells indicated that PPT displayed preferential affinity for zfERα than for zfERβ (Table 2 and Fig. 7). Similarly to phytoestrogens, known human ERβ selective synthetic agonists, such as DPN, 8β-VE2, FERB033, ERB033 and WAY200070 preferentially activated zfERα.

A recent study reported differences in ER subtype tissue localization in the developing zf larvae: zfERα is selectively expressed in the heart valves, zfERβ2 is robustly expressed in the liver, whereas zfERβ1 is not detected (Gorelik et al. 2014). In this same study, treatment of a transgenic zebrafish model containing a 5x consensus ERE sequence upstream of a GFP reporter (5xERE:GFP) with genistein, BPA and DPN preferentially induced GFP expression in the heart valves. PPT, on the other hand, showed GFP labeling of only the larval liver. Our study confirms the *in vivo* findings that these compounds present opposite selectivity of what has been observed for the human ER subtypes. Moreover, as the selectivity of several ligands is not conserved between human and zf ER subtypes, the determination of the transcriptional profile of estrogenic compounds towards the zfERs is crucial before their use *in vivo* to help elucidate distinct roles of each ER subtype in zf.

The hERs pan-antagonists ICI 182,780, 4-OH tamoxifen all acted as pure antagonists in the three zfERs. ICI 182,780 presented slight zfERα selectivity while 4-OH tamoxifen antagonized more efficiently zfERβ1 and zfERα than zfERβ2. In accordance with our results, ICI 182,780 blocks EE2-mediated transcriptional activation with all three isoforms of the zfERs in MDA-MB-231 cells transiently transfected with the zfERs (Notch and Mayer ; 2011). *In vivo,* treatment of zebrafish larvae with either ICI 182,780 or 4-OH tamoxifen reduce the E2-induced fluorescence in liver of transgenic 5XERE:GFP zebrafish (Gorelik and Halpern. 2011). Althrough ICI 182,780 has a higher antagonistic activity towards zfERα compared to zfERβ2 and β1, ICI 182,780 is also

efficient in reducing or blocking the GFP induced by various estrogens and pro-estrogens in the brain of cyp19a1b-GFP larvae (Brion et al. 2012). The hERα (raloxifen, MPP) and hERβ selective (PHTPP) antagonists were also tested on the HELN-zfERs reporter cells. Slight zfERα selectivity was also observed for PHTPP whereas raloxifen and MPP antagonized more efficiently zfERβ1 than zfERα and zfERβ2. Thus, there is also a non-conserved selectivity between zfERs and their human orthologs for antiestrogens.

To conclude, we have shown here that there are clear differences between the selectivity of various (anti)estrogens for zebrafish and human ER isoforms, establishing the fact that a direct translation of (anti)estrogenic effects (activities or potencies) from mammals to zebrafish is not possible. Although none of the tested compounds specifically activated either zebrafish or human ERs, the differences revealed in this study in terms of transcriptional activities towards human and zebrafish ERs highlight the need to take into account i) the species of origin and ii) the ER subtype when assessing the estrogenic potency of chemicals. This is particularly important in regards to EDCs screening for hazard assessment, since at the present time established test guidelines are only based on human cell lines expressing human estrogen receptors. To this end, such newly established in *vitro* cell lines together with those previously established in ZFL cells (Cosnefroy et al. 2012) can serve as useful screening tools to address estrogenic potency of chemicals for piscine models. Hence, an initial screening should be followed up with an ER-subtype specific analysis using both human and zebrafish ERs to elucidate the full spectrum of ER-mediated xenoestrogens effects.

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Figure legends

Figure 1. Zebrafish ERs are thermosensitive. Transcriptional activity of zfERα in response to different concentrations of E2 (A) and EE2 (B) was measured at 28, 31, 34 and 37°C. Transcriptional activity of zfERβ1 (C) and zfERβ2 (D) in response to E2 and EE2 were measured at 28 and 37°C. Results are expressed as % 10 nM E2. Standard deviations are from independent experiments done in quadruplicate.

Figure 2. Transcriptional activity of zfERα, zfERβ1 and zfERβ2 in response to endogenous estrogens. HELN-zfERα (■), HELN zf-ERβ1 (▲), HELN-zfERβ2 (○) cells were exposed to different concentrations of E1, E3 and 17αE2. Results are expressed as % of 10 nM E2 treatment. Standard deviations from at least 2 independent experiments done in quadruplicate are indicated.

Figure 3. Transcriptional activity of zfERα, zfERβ1 and zfERβ2 in response to myco and phytoestrogens. HELN-zfERα (■), HELN zf-ERβ1 (▲), HELN-zfERβ2 (○) cells were exposed to different concentrations of α-zea, genistein, liquiritigenin and ferutinin. Results are expressed as % of 10 nM E2 treatment. Standard deviations from at least 2 independent experiments done in quadruplicate are indicated.

Figure 4. Transcriptional activity of zfERα, zfERβ1 and zfERβ2 in response to environmental estrogens. HELN-zfERα (■), HELN zf-ERβ1 (▲), HELN-zfERβ2 (○) cells were exposed to different concentrations of BPA, 4-tet-octylphenol, NPm and BP2. Results are expressed as % of 10 nM E2 treatment. Standard deviations from at least 2 independent experiments done in quadriplicate are indicated.

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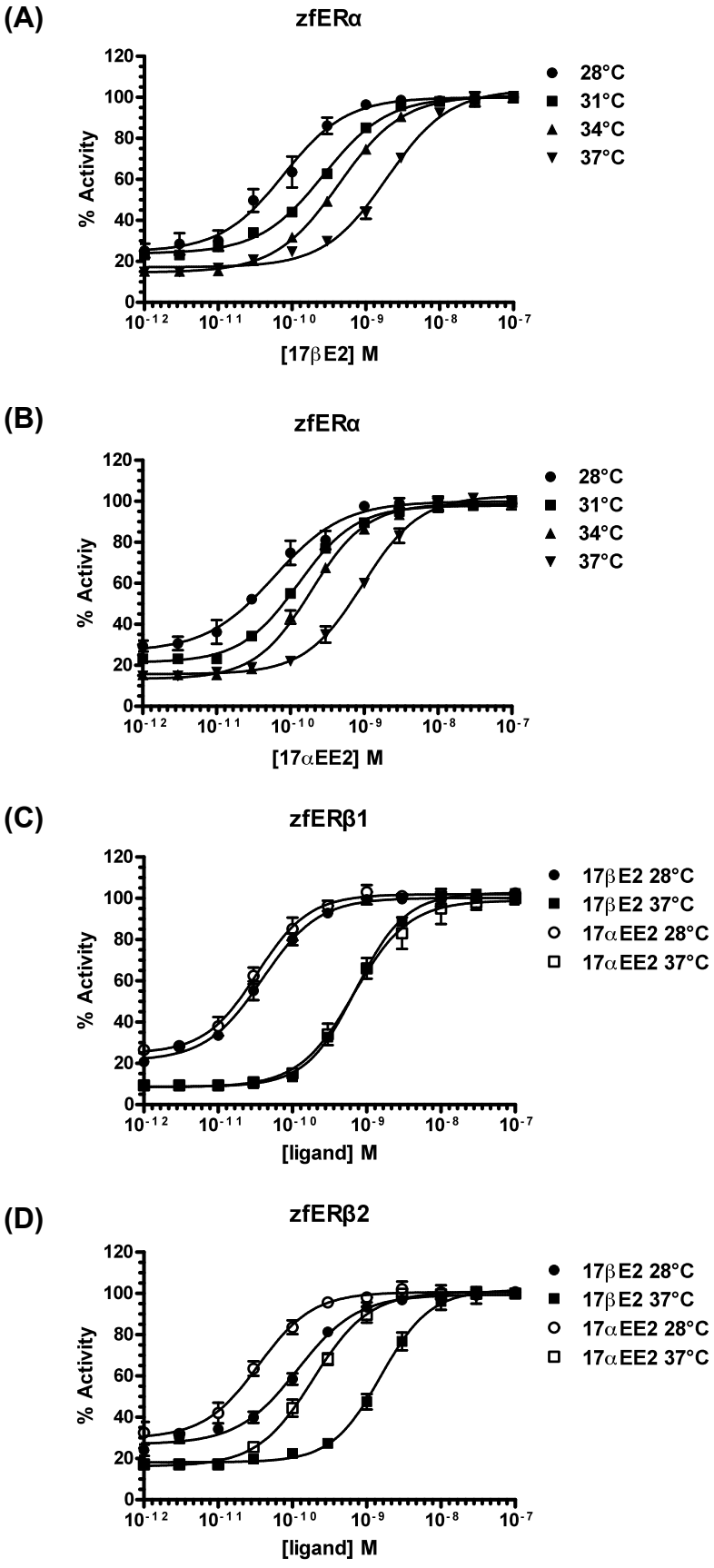
Figure 5. Transcriptional activity of zfERα, zfERβ1 and zfERβ2 in response to pharmaceuticals. HELN-zfERα (■), HELN zf-ERβ1 (▲), HELN-zfERβ2 (○) cells were exposed to different concentrations of 16αLE2, PPT, 8βVE2 and DPN. Results are expressed as % of 10 nM E2 treatment. Standard deviations from at least 2 independent experiments done in quadruplicate are indicated.

Figure 6. Transcriptional activity of zfERα, zfERβ1 and zfERβ2 in response to synthetic antagonists. HELN-zfERα (■), HELN zf-ERβ1 (▲), HELN-zfERβ2 (○) cells were exposed to different concentrations of ICI 182,780, 4OH-Tamoxifen, raloxifen and PPT together with 1 nM E2. Results are expressed as % of E2 treatment. Standard deviations from at least 2 independent experiments done in quadruplicate are indicated.

Figure 7. Amino acid alignment of human ERβ and zebrafish ERs ligand binding domains (LBDs) with par of the human ERα LBD (Met 342 to Ileu 426). The three amino acids involved in the binding of estrogens which differed betwen ERs are framed. Numbers above the human sequences indicate the number of amino acid residue within the total human ERα and ERβ sequences. The GenBank accession numbers of the ER sequences in this alignment are : human ERα NP_000116; human ERβ NP_001428; zebrafish ERα NP_694491; zebrafish ERβ1 CAC93848.1 and zebrafish ERβ2 CAC93849.1.

Figure 8. Modeling of the interaction between 16α-LE_2 and the human (h) and zebrafish (zf) estrogen receptors. Superposition of the structures of hERα (A) and hERβ (B) LBDs bound to E_2 (green) onto the molecular models of the 16α-LE_2-bound hERα (A) and 16α-LE_2-bound hERβ (B) LBDs (yellow). In hERα, the lactone ring of 16α-LE_2 points towards M421 (H7) which undergoes a large conformational change (black arrow) to accommodate this additional group. In

hERβ, the linear M421 is replaced by the branched residue I373 which is characterized by a much smaller intrinsic flexibility that maintains the synthetic ligand in a position (black arrow) where it interacts unfavorably with M336 (H3, red asterisk). Key hydrogen bonds between the protein and the ligands are highlighted by doted lines. W denote a water molecule. Superposition of the structures of hERα (C) and hERβ (D) LBDs bound to E_2 (green) onto the molecular models of the 16α-LE_2-bound zfERα (C) and 16α-LE_2-bound zfERβ1 or zfERβ2 (yellow and violet, respectively) (D) LBDs. The steric constrains observed in hERs also apply to zfERs and account for the differential affinity of 16α-LE_2 for the zfER subtypes. In (C) the doted line highlight a stabilizing interaction between hERα and the ligand that is probably missing in zfERα.


(A)
zfERα
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28°C
31°C
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17αEE2 37°C
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Figure 1

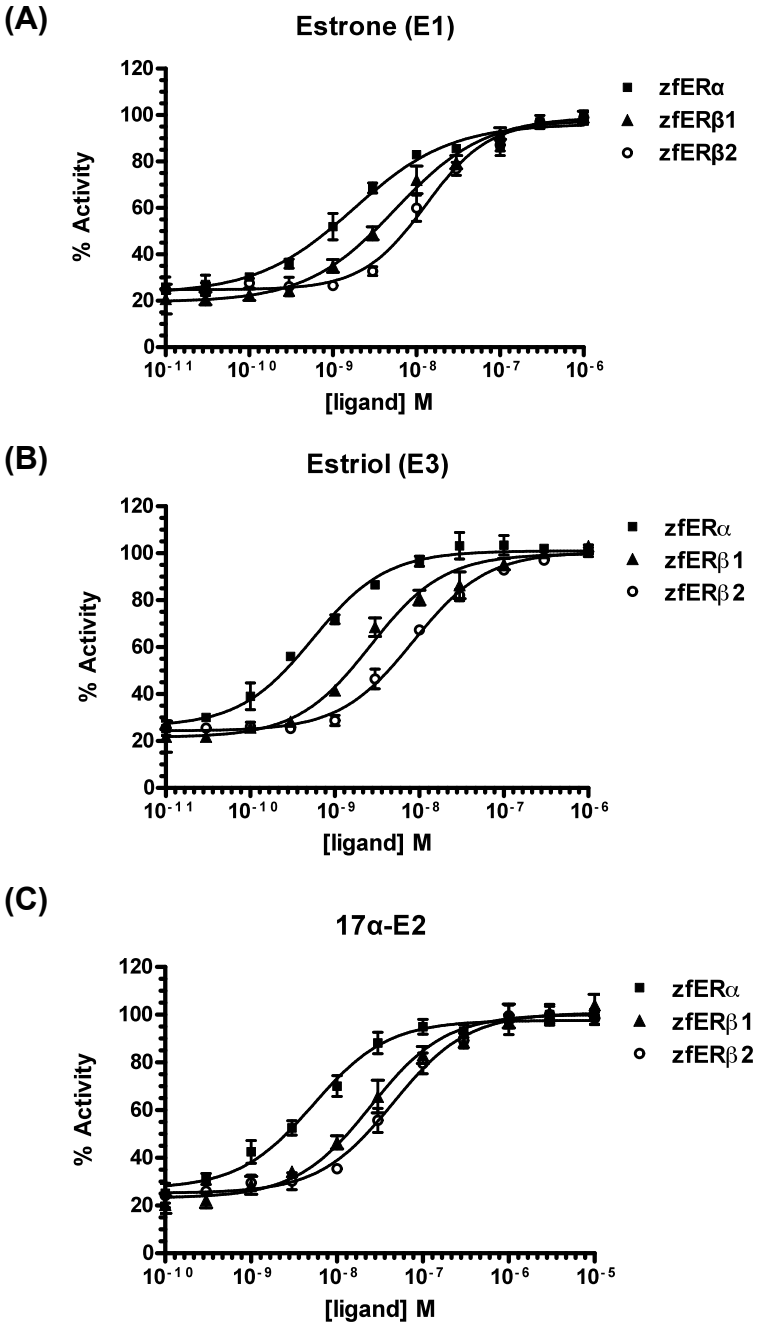
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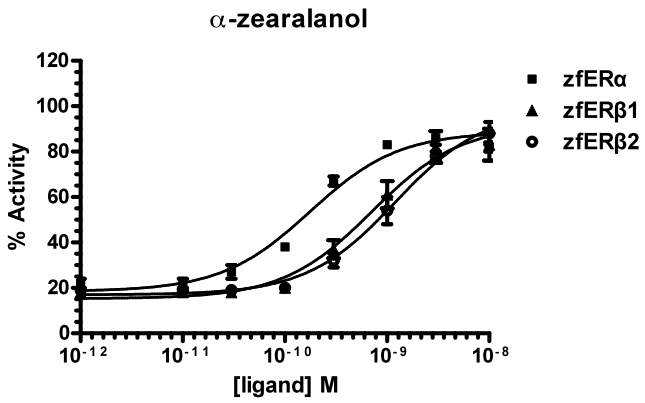
Figure 2

(A)



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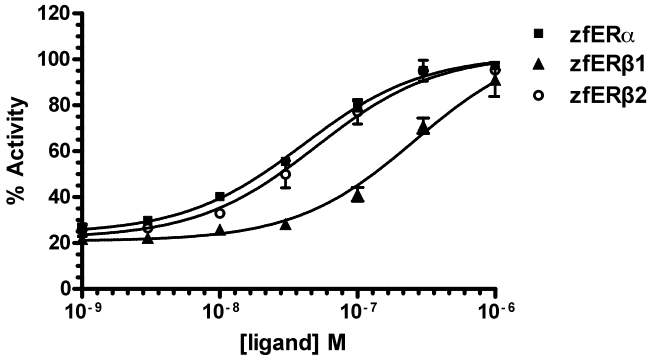

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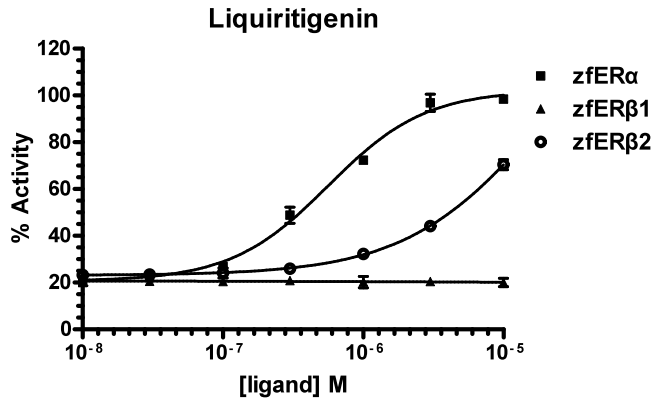

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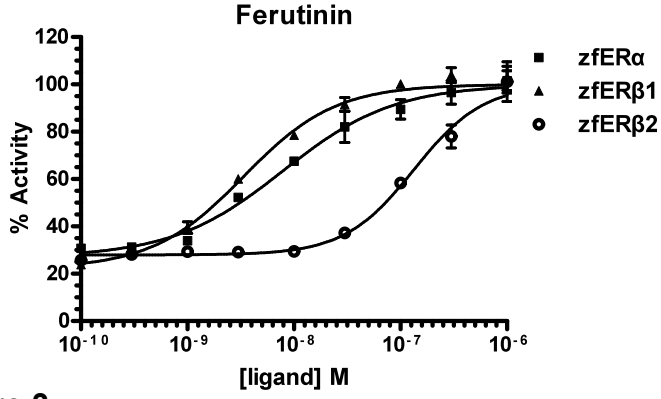
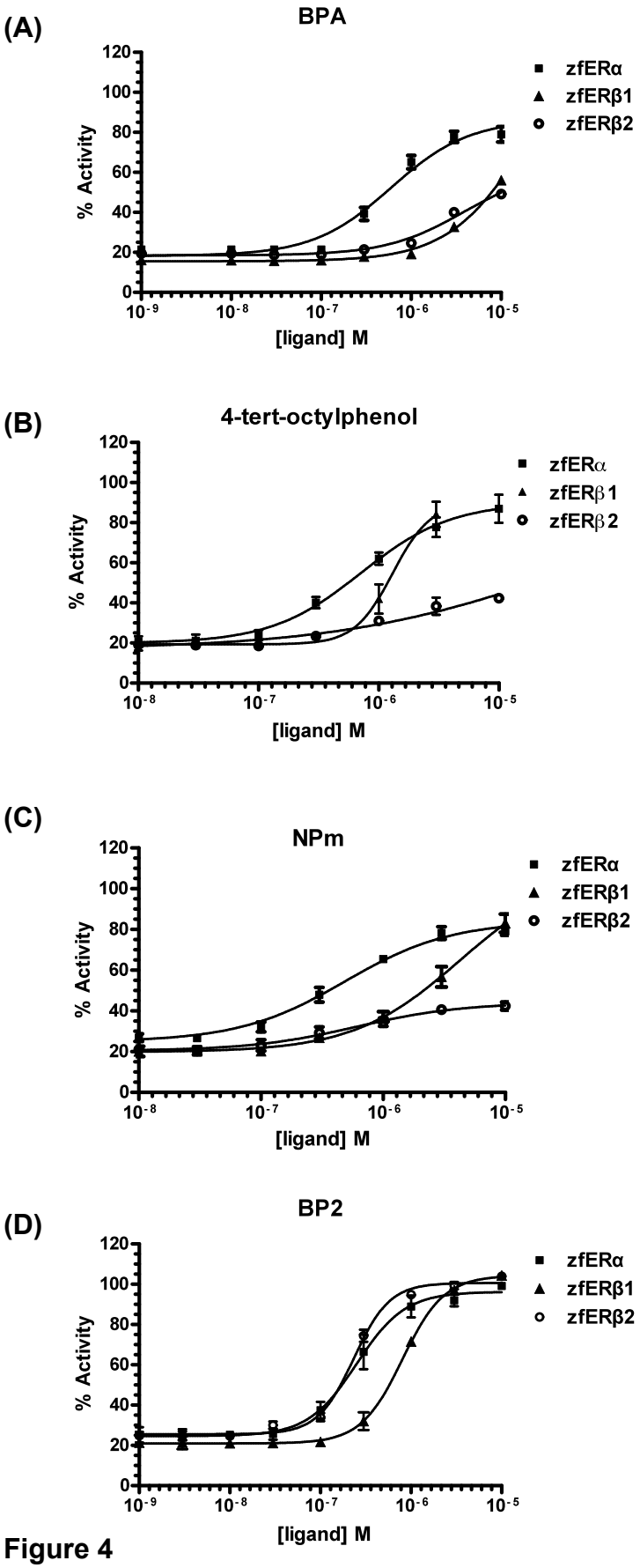


Figure 3


(A)
BPA
% Activity
[ligand] M
zfERα
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(B)
4-tert-octylphenol
% Activity
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zfERα
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zfERβ2
(C)
NPm
% Activity
[ligand] M
zfERα
zfERβ1
zfERβ2
(D)
BP2
% Activity
[ligand] M
zfERα
zfERβ1
zfERβ2

Figure 4

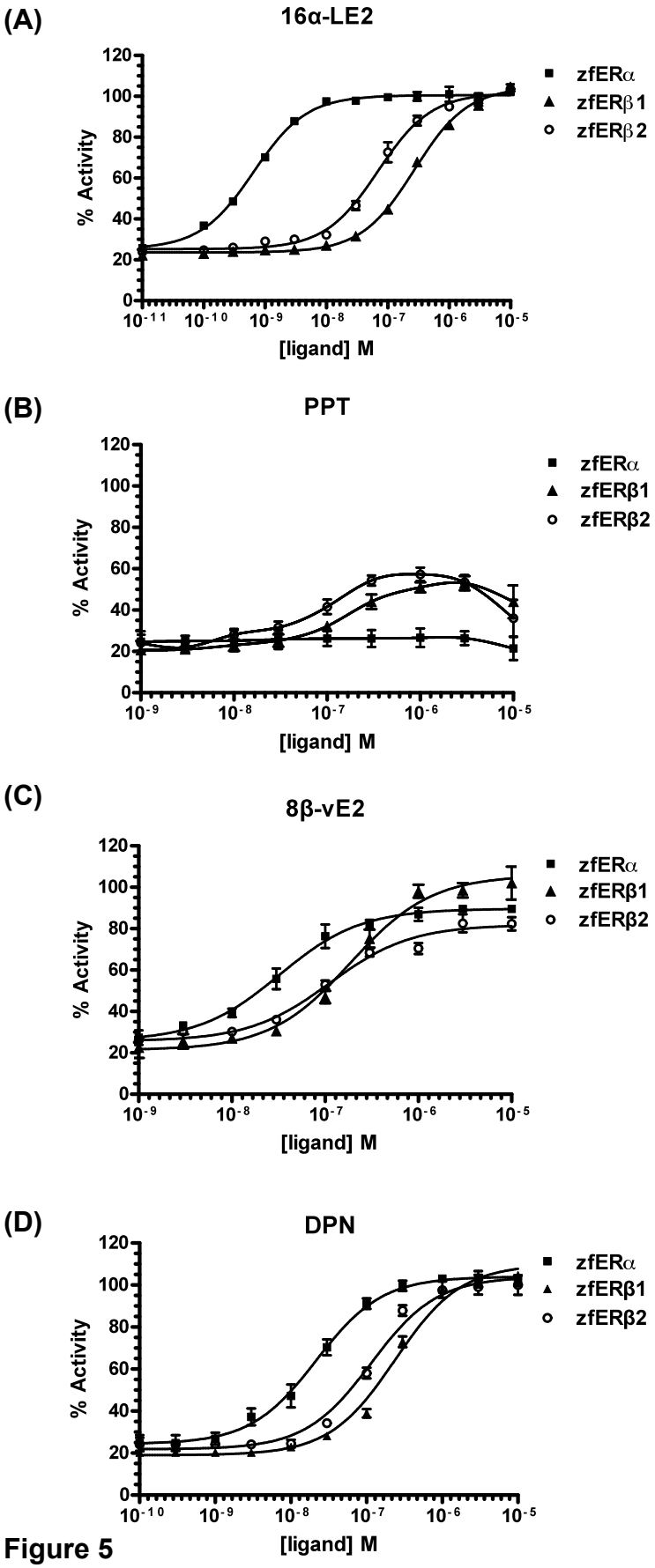
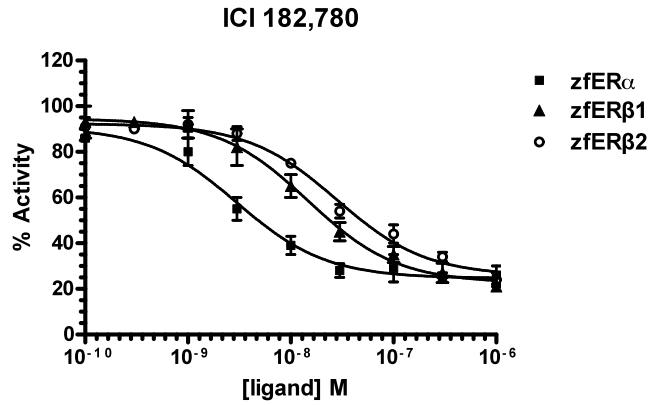
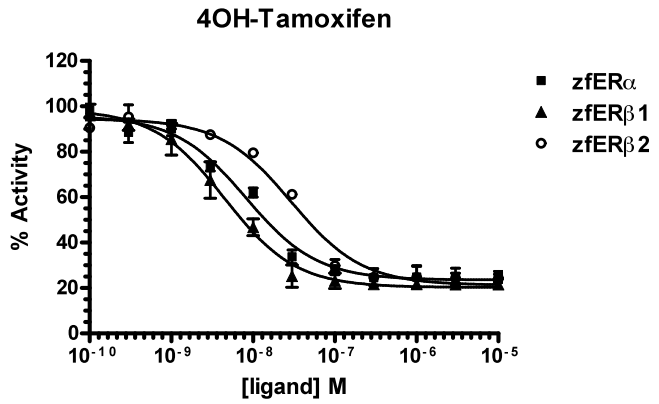

(A)
16α-LE2
% Activity
[ligand] M
zfERα
zfERβ1
zfERβ2
(B)
PPT
% Activity
[ligand] M
zfERα
zfERβ1
zfERβ2
(C)
8β-vE2
% Activity
[ligand] M
zfERα
zfERβ1
zfERβ2
(D)
DPN
% Activity
[ligand] M
zfERα
zfERβ1
zfERβ2

Figure 5

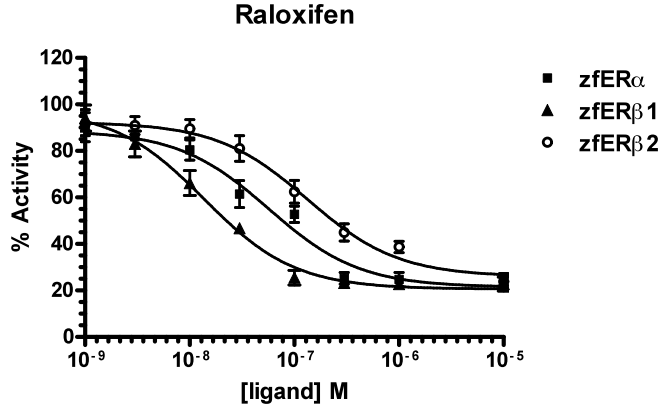
(A)



(B)



(C)



(D)

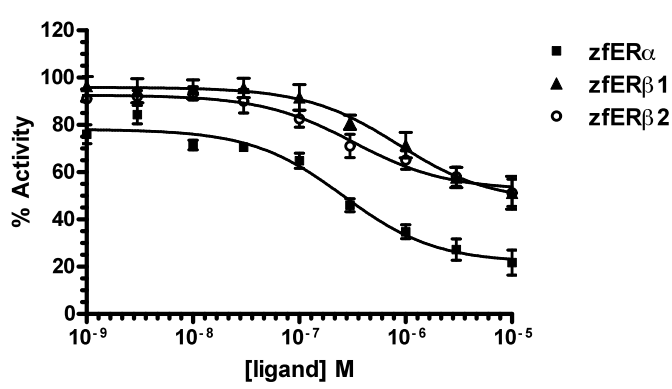


Figure 6

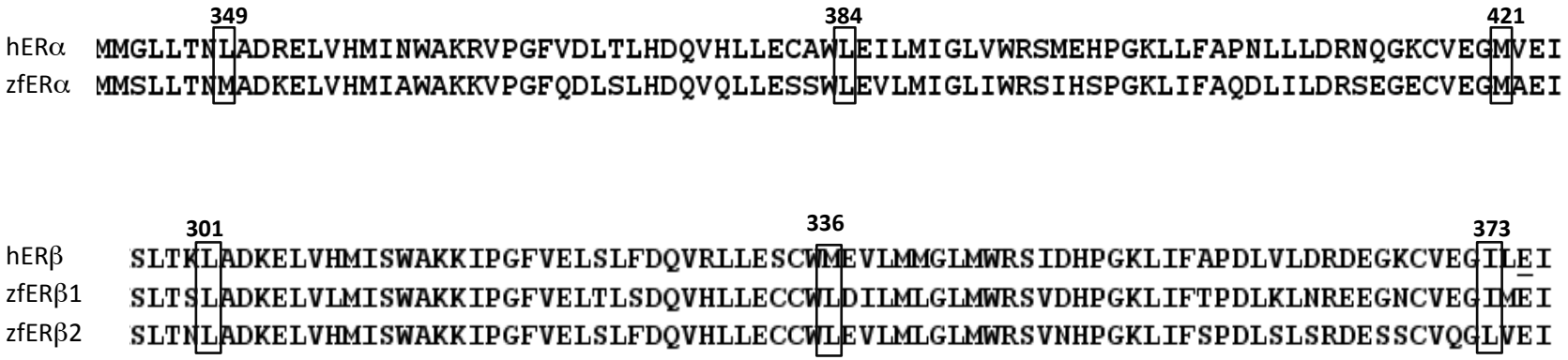

349
384
421
hERα MMGLLTNLADRELVHMINWAKRVPGFVDLTLHDQVHLLECAWLEILMIGLVWRSMEHPGKLLFAPNLLLDRNQGKCVEGMVEI
zfERα MMSLLTNMADKELVHMIAWAKKVPGFQDLSLHDQVQLLESSWLEVLMIGLIWRSIHSPGKLIFAQDLILDRSEGECVEGMAEI
301
336
373
hERβ SLTKLADKELVHMISWAKKIPGFVELSLFDQVRLLESCWMEVLMMGLMWRSIDHPGKLIFAPDLVLDRDEGKCVEGILEI
zfERβ1 SLTSLADKELVLMISWAKKIPGFVELTLSDQVHLLECCWLDILMLGLMWRSVDHPGKLIFTPDLKLNREEGNCVEGIMEI
zfERβ2 SLTNLADKELVHMISWAKKIPGFVELSLFDQVHLLECCWLEVLMLGLMWRSVNHPGKLIFSPDLSLSRDESSCVQGLVEI

Figure 7

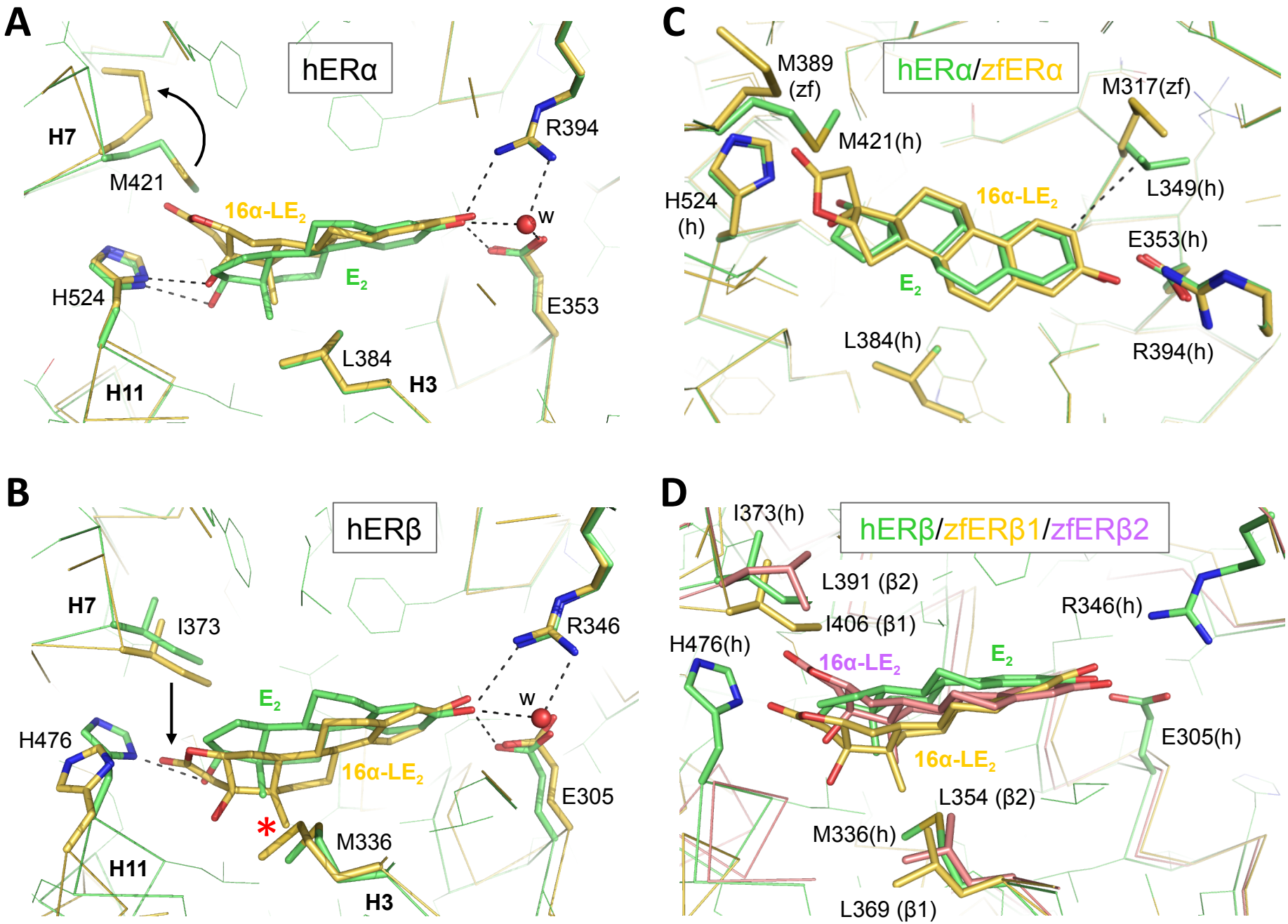

A
hERα
H7
M421
16α-LE2
R394
w
E2
H524
E353
L384
H3
H11
C
hERα/zfERα
M389
(zf)
M317(zf)
M421(h)
H524
(h)
16α-LE2
L349(h)
E353(h)
E2
L384(h)
R394(h)
B
hERβ
H7
I373
R346
E2
w
H476
16α-LE2
E305
M336
H11
H3
D
I373(h)
hERβ/zfERβ1/zfERβ2
L391 (β2)
R346(h)
I406 (β1)
H476(h)
E2
16α-LE2
E305(h)
16α-LE2
L354 (β2)
M336(h)
L369 (β1)

Figure 8