BIOCHEMICAL PHARMACOLOGY 71 (2006) 1459–1469

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Evaluation of ligand selectivity using reporter cell lines stably expressing estrogen receptor alpha or beta

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ARTICLE INFO

Article history:
Received 30 November 2005
Accepted 1 February 2006

Keywords:
Estrogen receptors
Ligand
Stable transfectants
Whole-cell ligand binding
Transactivation

Abbreviations:
AF, activation function
DCC, dextran-coated charcoal
DMEM, Dulbecco's modified Eagle's medium
DMSO, dimethyl sulfoxide
EC50 value, efficient concentration to obtain half maximum activity
ER, estrogen receptor
ERE, estrogen-responsive element
HELN, human cervix adenocarcinoma cell expressing luciferase under estrogen-response element control
IC50 value, concentration required to inhibit specific E2 binding by 50%
K_d, dissociation constant

ABSTRACT

Estrogens control transcriptional responses through binding to two different nuclear receptors, estrogen receptor α (ERα) and β (ERβ). Since these two ER subtypes are thought to mediate different biological effects, there is intense interest in designing subtype-selective ER ligands. In this study, we evaluated the ERα and ERβ selectivity of 19 known estrogens and antiestrogens using reporter cell lines previously developed in our laboratory. The HELN-ERα and HELN-ERβ cells stably express full-length ERα and ERβ, respectively, and are derived from HELN cells (HeLa cells stably transfected with an ERE-driven luciferase plasmid). We report that 16α-LE2, PPT and 3β,5α-GSD have a high ERα-selective agonist potency while 8β-VE2, DPN, genistein and biochanin A show ERβ selectivity with 8β-VE2 being the most potent and selective ERβ agonist. We also tested ER antagonists and we showed that raloxifene and RU486 are ERα and ERβ-selective antiestrogens, respectively. In all cases, selectivity is due to differences in binding affinities as indicated by whole-cell ligand-binding assays. Very interestingly, we demonstrate that a combination of genistein and raloxifene produces a full-ERβ specific response. Together these results demonstrate the usefulness of our stably transfected cell lines to characterize ER ligands and indicate that treatments combining agonist/antagonist ligands produce full-ERβ selectivity.

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0006-2952/$ – see front matter
doi:10.1016/j.bcp.2006.02.002

LBD, ligand-binding domain
Luc, firefly luciferase
RLU, relative luciferase units
RBA, relative binding affinity
RTC, relative transactivation capacity
SERM, selective estrogen receptor modulators

1. Introduction

Estrogens regulate important physiological processes in a large number of target tissues, including uterus, breast, bone, brain, and cardiovascular system. Therefore, estrogenic pharmaceuticals have been developed to treat pathologies such as infertility, breast cancer, and osteoporosis [1]. Many synthetic ligands used in hormone therapy are known as selective estrogen receptor modulators (SERMs). This reflects their ability to behave as estrogen agonists in some tissues while behaving as antagonists in others [2].

The action of estrogens in regulating gene transcription is mediated through specific estrogen receptors (ERs), which are members of the nuclear receptor superfamily [3]. All members of this superfamily have a similar architecture. The amino-terminal A/B domain is involved in transactivation of gene expression. The central C-domain contains a two-zinc finger structure, which plays an important role in specific binding to DNA and in receptor dimerization. The carboxyl-terminal domain (or E/F domain) is crucial for ligand binding, nuclear translocation, receptor dimerization, and modulation of target gene expression in association with coactivators and corepressors [4]. The transactivation functions of both the A/B (AF-1) and the E/F (AF-2) domains depend on promoter context and cell type [5]. In the classical way of action, the 17-β estradiol (E2)-activated receptor directly binds DNA on so-called estrogen-response elements. It then recruits different type of transcription coactivators which either modify chromatin structure or serve as a bridge between the receptor and the general machinery. However, the interaction of ER with target genes can occur indirectly through recruitment by transcription factors such as AP-1 or Sp1 [6].

Two estrogen receptor subtypes (ERα and ERβ), encoded by different genes, were identified [7,8]. Mice lacking one or both receptors were created in an attempt to define their functions. The ERα knock-out mice are infertile (male and female), they have decreased bone density and a disturbed breast development. The ERβ knock-out mice develop normally but females have very reduced fertility due to defects in both ovary and uterus [9–11].

Although both estrogen receptor subtypes are activated upon E2 binding, they have different primary sequences in their AF-2 containing hormone binding domains which show 56% amino acid identity [12,13]. As a consequence, some ER subtype-selective ligands have been identified. These ligands present binding affinities different for the two receptors and exhibit variable agonistic or antagonistic characters according to the ER considered. Because ERα and ERβ have distinct physiological functions, selective ligands could display very specific biological responses at the tissue level and therefore improve hormonal therapy of ER-dependent diseases [14].

Quite diverse compounds (estrogens, antiestrogens, phytoestrogens and synthetic estrogens) have been identified in several laboratories, some of which present a subtype-selective affinity [15]. In this study, we used stably transfected HELN-ERα or -ERβ responsive reporter cell lines to test compounds exhibiting ER subtype-selective activities. These stable cell lines enabled us to characterize the ''whole-cell'' affinity of ligands for ERα and ERβ and to precisely compare their effects on transcriptional activation.

2. Materials and methods

2.1. Materials

Materials for cell culture came from Life Technologies (Cergy-Pontoise, France). Luciferin (sodium salt) was purchased from Promega (Charbonnières, France). [2,4,6,7,16,17-^{3}H]-E2 (41.3 Ci/mmol specific activity) was purchased from NEN Life Science Products (Paris, France). 17-β Estradiol (E2) (1,3,5[10]-estratriene-3,17β-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 (4′,5,7-trihydroxyisoflavone), biochanin A (5,7-dihydroxy-4′-methoxyisoflavone), daidzein (4′,7-dihydroxyisoflavone), coumestrol (2-(2,4-dihydroxyphenyl)-6-hydroxy-3-benzofuran carboxylic acid lactone), were purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France). 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 from Zeneca (Macclesfield, UK). Raloxifene (6-hydroxy-3-[4-[2-(1-piperidinyl)ethoxy]phenoxy]-2-(4-hydroxy phenyl)-benzothiophene) was from Eli Lilly (Indianapolis, USA). RU486 ((11β,17β)-11-[4-(dimethylamino)phenyl]-17-hydroxy-17-(1-propynyl)-estra-4,9-dien-3-one) was from Sanofi-Aventis (Romainville, France). 3β,5α-GSD, 3β,5α-LNG and 3β,5α-NET were synthesized as already described [16]. 4,4′,4″-(4-Propyl-[^{1}H]-pyrazole-1,3,5-triyl)trisphenol (PPT) and 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN) were purchased from Tocris (Ellisville, 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 were purchased from Research Laboratories of Schering AG (Berlin, Germany). These ligands were dissolved in dimethyl sulfoxide (DMSO) at 10^{-2} M. Successive dilutions were performed in culture medium.

2.2. Cell lines

The stably transfected cell lines were already described [17]. Briefly, to generate HELN cells, we transfected HeLa cells with the ERE-βGlobin-Luc-SVNeo plasmid [18]. HELN-ERα and HELN-ERβ cell lines were obtained by a second transfection of the corresponding pSG5-puro plasmids (pSG5-ERα-puro, pSG5-ERβ-puro, respectively). Selection by geneticin and puromycin was done at 1 mg/ml and 0.5 μg/ml, respectively. Luminescent and inducible clones were identified with a photon counting camera (Argus 100 from Hamamatsu, Japan or NightOWL LB 981 from Berthold Technologies, Bad Wildbad, Germany) and the most responsive clones were isolated.

2.3. Cell culture conditions

For the strain culture, HELN cell line was grown in phenol red containing Dulbecco's Modified Eagle's Medium (DMEM), 1 g/l glucose, supplemented with 5% of fetal calf serum (FCS) and 1% antibiotic (penicillin/streptomycin) in a 5% CO_2 humidified atmosphere at 37 °C. Because of phenol red and FCS estrogenic activity, HELN-ERα and HELN-ERβ cell lines were grown in phenol red-free medium supplemented with 6% dextran-coated charcoal (DCC)-treated FCS and 1% antibiotic (penicillin/streptomycin) (6% DCC-FCS). Experiments were achieved in this 6% DCC-FCS medium.

2.4. ERα and ERβ stable transactivation assays

HELN-ERα and HELN-ERβ cells lines were seeded at a density of 4×10^4 cells/well in 96-well white opaque tissue culture plates (Greiner CellStar, D. Dutscher, Brumath, France) and maintained in 6% DCC-FCS. Compounds to be tested were added 8 h later and cells incubated with compounds for 16 h. Experiments were performed in quadruplicate and repeated three times. At the end of the incubation, effector containing medium was removed and replaced by 0.3 mM luciferin containing 6% DCC-FCS. At this concentration, luciferin diffuses into the cell and produces a luminescent signal that is stable from 5 min on. It is approximately 10-fold less intense than a signal after cell lysis would be, but it is perfectly stable for several hours. The 96-well plate was then introduced in a microplate luminometer (Microbeta, Wallac) and intact living cell luminescence measured for 2 s. EC50 values were evaluated using Graph-Pad Prism statistics software (version 4.0; Graphpad Software Inc., San Diego, CA, USA).

2.5. Ligand-binding analysis

HELN-ERα or HELN-ERβ cells were seeded at a density of 10^5 cells/well in 24-well tissue culture plates and grown in 6% DCC-FCS. Cells were labeled with [^{3}H]-E2 (41.3 Ci/mmol specific activity) at 37 °C for 16 h in the absence or presence of non-radioactive E2 or competitive compounds. The final incubation volume was 400 μl and each dilution was performed in duplicate. After incubation, unbound material was aspirated and cells washed three times with 400 μl of cold PBS. Then, 250 μl lysis buffer (400 mM NaCl, 25 mM Tris phosphate pH 7.8, 2 mM DTT, 2 mM EDTA, 10% glycerol, 1% triton X-100) was added and plates were shaked for 5 min. Total cell lysate (200 μl) was mixed with 4 ml of LSC-cocktail (Emulsifier-Safe, Packard BioScience) and [^{3}H] bound radioactivity was liquid scintillation counted (LS-6000-SC, Beckman-Coulter, Roissy, France). Protein concentrations were measured by Bio-Rad protein assay and used to normalize bound radioactivity values expressed in dpm.

For saturation ligand-binding analysis experiments, cells were labeled with 0.01–3 nM [^{3}H]-E2 in the absence or presence of 100 nM of non-radioactive E2. Specific binding was determined by subtracting non-specific binding from total binding. Free ligand concentration was calculated by subtracting bound ligand from total ligand. The dissociation constant (K_d) value was calculated as the free concentration of radioligand at half-maximal binding by fitting data to the Hill equation and by linear Scatchard transformation.

For ligand competition experiments, each compound was tested at least three times. Cells were labeled with 0.1 nM [^{3}H]-E2 (in the absence or presence of increasing concentrations of non-radioactive competitive compounds). In absence of competitor, specific bound radioactivity was 750–1000 dpm. Results were plotted as measured dpm versus concentration of tested compound. IC50 values were defined as compound concentration required to decrease maximum [^{3}H]-E2 binding by 50%. Compound selectivity toward ERα and ERβ was evaluated using the relative binding affinity (RBA) to E2. RBA for each competitor was calculated as the ratio of E2 to competitor concentration required to reduce specific radioligand binding by 50% (=ratio of IC50 values). The RBA value for E2 was arbitrarily set at 100.

3. Results

3.1. Characterization of stable HELN-ERs reporter cell lines

These reporter cell lines were previously established in our laboratory [18]. Because it does not express endogenous ERs, the human cervix adenocarcinoma HeLa cell line was chosen as a host to generate stable reporter cell lines to screen compounds that act via human ERα and/or ERβ. Generation of the HELN-ERα and HELN-ERβ reporter cell lines was performed in two steps. An estrogen-responsive reporter gene was first stably introduced into HeLa cells, generating the HELN cell line. The reporter gene ERE-βGlobin-Luc-SVNeo contains a luciferase gene driven by an estrogen-responsive element (ERE) in front of the βGlobin promoter and a neomycin phosphotransferase gene under the control of the SV40 promoter [18]. In a second step, HELN cells were transfected with an ERα or ERβ expression plasmid to obtain the HELN-ERα and HELN-ERβ cell lines, respectively.

As shown in Fig. 1A, receptor protein level (ERα or ERβ) expressed in each cell line was determined by saturation ligand-binding assay with [^{3}H]-E2 in a "whole-cell" experiment (see Section 2). HELN-ERα and HELN-ERβ expressed 52.6 and 43.1 fmol of ER per mg of protein, respectively. K_d values, calculated from saturation curves, were 0.04 and 0.11 nM for ERα and ERβ, respectively (Fig. 1A). These K_d values were within the range generally reported for E2 binding to ERs in various systems [19].

As previously reported, HELN-ERs cell lines showed E2-mediated luciferase gene transactivation whereas HELN cell

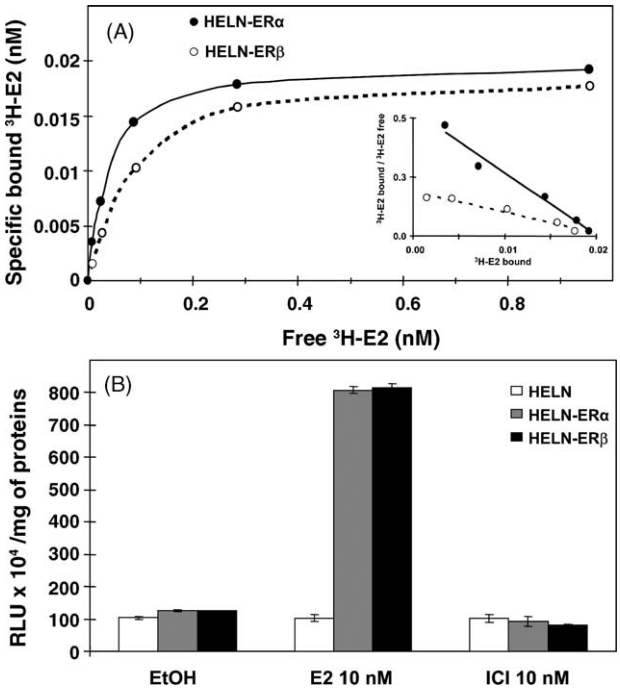

Fig. 1 – Characterization of the HELN-ERα and HELN-ERβ stably transfected cell lines. (A) Binding of [^{3}H]-E2 to ERα (●) and ERβ (○) in presence or absence of an excess of E2. Specific bound counts were calculated by subtracting non-specific counts from total bound counts. Inset, Scatchard analysis of specific binding giving a K_d of 0.04 and 0.11 nM for ERα and ERβ, respectively. (B) Stable luciferase gene expression in the HELN, HELN-ERα and HELN-ERβ cell lines. Relative light units were normalized by mg of protein. Results are expressed as 10^4 RLU/mg of proteins.

luciferase expression was not modulated (Fig. 1B). The basal activity of HELN-ERα and HELN-ERβ (105 and 124 × 10^4 RLU/mg of protein, respectively) was slightly reduced in presence of ICI 182,780 pure antiestrogen (81 and 61 × 10^4 RLU/mg of protein, respectively). As also shown in Fig. 1B, E2-mediated reporter gene activities through ERα and ERβ were comparable (880 and 910 × 10^4 RLU/mg of protein, respectively). These results are in agreement with those obtained by Delaunay et al., who found that ERβ was as transcriptionally active as ERα in transiently transfected HeLa cells [20]. The HELN-ERα and HELN-ERβ cell lines appeared therefore as ideal tools to compare ligand different effects toward the two isoforms both in term of binding to receptors and ability to regulate their transcriptional activity.

3.2. *Comparison of ligand-binding affinities for ERα and ERβ*

Nineteen compounds were tested with both ER subtypes in ''whole-cell'' competition experiments performed with 100 pM [^{3}H]-E2 in HELN-ERα and HELN-ERβ cell lines. We used IC50 values to calculate relative binding affinities (RBA). RBA values for E2 are arbitrarily set at 100 for each receptor (IC50 of 0.12 nM for ERα, IC50 of 0.18 nM for ERβ). RBA values for all tested compounds are shown in Table 1.

Among natural estrogens, we tested estrone (E1) and estriol (E3) since these metabolic products of E2 are still capable of exerting various biological actions. The relative binding affinity of E1 for both ERα and ERβ differed only slightly (RBA of 4 and 3.5 for ERα and ERβ, respectively). By contrast, E3 bound preferentially ERβ, as shown by an ERα/ERβ RBA ratio of 0.64.

Table 1 – Binding affinity of compounds for ERα and ERβ in whole cells

Compounds	RBA ERα ± S.D.	K_i ERα (nM)	RBA ERβ ± S.D.	K_i ERβ (nM)	RBA ERα/ERβ
Estradiol	100 ± 0	0.04	100 ± 0	0.11	1.00
Estrone	4.0 ± 1.1	1.01	3.5 ± 0.12	3.1	1.1
Estriol	11.3 ± 2.9	0.35	17.6 ± 0.61	0.63	0.64
Genistein	0.032 ± 0.002	126	0.86 ± 0.058	12.8	0.037
Biochanin A	0.022 ± 0.003	174	1.2 ± 0.40	8.9	0.018
Coumestrol	0.05 ± 0.010	80.0	0.41 ± 0.081	27.0	0.12
Daidzein	0.015 ± 0.003	262	0.13 ± 0.034	85.3	0.12
Ethynylestradiol	233 ± 41	0.02	37.8 ± 5.1	0.29	6.2
3β,5α-GSD	0.5 ± 0.001	8.0	0.007 ± 0.001	1553	70.6
3β,5α-LNG	0.3 ± 0.001	13.3	0.018 ± 0.004	629	17.1
3β,5α-NET	2.08 ± 0.59	1.92	0.058 ± 0.012	189	35.7
PPT	10.0 ± 2.8	0.40	0.12 ± 0.009	92.8	84.4
16α-LE2	14.6 ± 1.9	0.27	0.089 ± 0.038	131	164
DPN	0.12 ± 0.02	32.4	6.6 ± 0.53	1.7	0.019
8β-VE2	0.35 ± 0.02	12.9	22.0 ± 2.8	0.50	0.016
ICI 182,780	9.4 ± 2.4	0.42	8.3 ± 6.4	1.3	1.1
4OH-Tam	1.7 ± 0.5	2.3	2.3 ± 0.74	4.8	0.74
Raloxifene	7.8 ± 3.2	0.52	0.54 ± 0.24	20.2	14.2
RU486	0.0055 ± 0.001	727	0.030 ± 0.001	367	0.18

Whole-cell ligand competition assays were performed using 0.1 nM [^{3}H]-E2 as tracer. RBA of each competitor was calculated as ratio of E2 or competitor concentration required to reduce the specific radioligand binding by 50%. RBA is relative binding affinity where E2 = 100. K_i values were calculated from the RBA values and the K_d values for E2 using the Cheng-Prusoff relationship [38].

Phytoestrogens are plant constituents exhibiting structural similarities with endogenous estrogens. Biochanin A and its metabolite genistein, coumestrol and daidzein had a higher binding affinity for ERβ than for ERα. ERβ/ERα RBA ratios for coumestrol, daidzein and genistein were in agreement with previously described data [19], genistein and biochanin A displaying the largest ERβ selectivity (ERα/ERβ RBA of 0.018).

We also tested synthetic ligands like 17α-ethynylestradiol (EE2) and three 3β,5α-tetrahydro derivatives of norethisterone (NET), of gestodene (GSD) and of levonorgestrel (LNG). The RBA of EE2 for ERα was six-fold-higher than for ERβ. The A ring reduced metabolites of 19-nor synthetic progestins (3β,5α-GSD, 3β,5α-LNG and 3β,5α-NET) had also a better binding affinity for ERα than that for ERβ, and particularly 3β,5α-GSD which exhibited an ERα/ERβ RBA ratio of 70.

We next tested commercially available ligands such as 4-propyl-1,3,5-tris(4hydroxyphenyl) pyrazole (PPT), diarylpropionitrile (DPN), and two estradiol derivatives, estradiol 16α-lactone (16α-LE2) and 8β-vinyl estradiol (8β-VE2). All of them have been described as ER-subtype-selective ligands [21]. The member of the triarylpyrazole class (PPT) was found to have a strong affinity for ERα (RBA of 10) and an ERα subtype-selectivity (ERα/ERβ RBA ratio of 85). Compounds with larger 16α substitutions such as 16α-LE2 showed a similar affinity (IC50 of 0.8 nM) and a stronger selectivity (ERα/ERβ RBA ratio of 165) for ERα. The non-steroidal compound, DPN showed an ERβ binding selectivity similar to biochanin A (ERα/ERβ RBA ratio of 0.019). Larger substitutions in position 8β increased ERβ selectivity, as shown with 8β-VE2 which exhibited an IC50 of 0.8 nM, and an ERα/ERβ RBA ratio of 0.016 (substantially higher than that of genistein and biochanin A).

Among antagonists, 4-hydroxytamoxifen (4OH-Tam), ICI 182,780, raloxifene and the antiprogestin RU486 were tested. Raloxifene displayed the highest selectivity for ERα (ERα/ERβ RBA ratio of 14) whereas RU486 was the most selective antiestrogen towards ERβ (ERα/ERβ RBA ratio of 0.18).

3.3. Analysis of natural agonist ligand selectivity on transcriptional activity

We first compared natural estrogen ability to increase transactivation in HELN-ERα and HELN-ERβ cell lines (Fig. 2). EC50 values and relative transactivation capacities (RTC) for all tested compounds are recapitulated in Table 2. As previously reported [18], EC50 value for E2 was approximately 0.017 nM for ERα and 0.068 nM for ERβ. E1 and E3 displayed a slight ERβ selectivity with a 2.4-fold and a 2.6-fold higher potency, respectively.

We then measured phytoestrogen effects (Fig. 3). Genistein, biochanin A and coumestrol had the same agonistic activity on ERβ (EC50 values of 5.8, 6.8 and 6.9 nM, respectively), whereas daidzein had a very low agonistic activity on ERβ (EC50 value of 57 nM). However, biochanin A and genistein, with a 12-fold and a 6.6-fold higher potency to transactivate luciferase gene expression, were the best ERβ selective compounds. Genistein demonstrated a full agonism in HELN-ERα and HELN-ERβ cell lines with EC50 values of 38 nM for ERα and 5.8 nM for ERβ. It is interesting to note that Barkhem et al. demonstrated that genistein was a partial agonist on ERβ in 293/hERβ cells indicating that the activity of genistein-liganded ERβ is certainly cell-dependent [22].

Moreover, at concentration higher than 1 μM, we noticed an overactivation of the luciferase reporter gene by genistein, daidzein and biochanin A which was observed not only in HELN-ERα and HELN-ERβ cells but also in the parental HELN cell line (Fig. 3 and data not shown). This effect, which was previously reported for genistein [19], indicated that luciferase expression obtained at high concentrations of phytoestrogens needs to be examined carefully.

3.4. Effect of synthetic ligands on ERα and ERβ transcriptional activities

We next examined the ability of synthetic ligands such as EE2, 3β,5α-NET, 3β,5α-GSD and 3β,5α-LNG to activate transcription

Table 2 – Effect of compounds on ERα and ERβ transactivation in whole cells

Compounds	EC50 (nM) ERα ± S.D.	RTC ERα	EC50 (nM) ERβ ± S.D.	RTC ERβ
Estradiol	0.017 ± 0.003	100	0.068 ± 0.011	100
Estrone	0.66 ± 0.12	2.6	1.6 ± 0.3	4.3
Estriol	0.16 ± 0.02	10.6	0.41 ± 0.09	16.6
Genistein	38 ± 16	0.045	5.8 ± 1.6	1.2
Biochanin A	82 ± 27	0.021	6.8 ± 1.9	1.0
Coumestrol	16 ± 3	0.11	6.9 ± 1.3	1.0
Daidzein	150 ± 46	0.011	57 ± 17	0.12
Ethynylestradiol	0.008 ± 0.003	213	0.25 ± 0.06	27.2
3β,5α-GSD	5.6 ± 1.1	0.30	4100 ± 1600	0.002
3β,5α-LNG	15 ± 4	0.11	5100 ± 2500	0.001
3β,5α-NET	1.3 ± 0.5	1.3	110 ± 29	0.062
PPT	0.085 ± 0.024	20.0	ND	ND
16α-LE2	0.09 ± 0.015	18.9	93 ± 16	0.07
DPN	27 ± 8	0.063	2.3 ± 0.3	3.0
8β-VE2	15 ± 5	0.11	0.2 ± 0.1	34.0

Relative transactivation capacity (RTC) of each competitor was calculated as ratio of concentrations of E2 or competitor required to reduce the specific transactivation by 50% (=ratio of EC50 values). RTC value for E2 was arbitrarily set at 100.

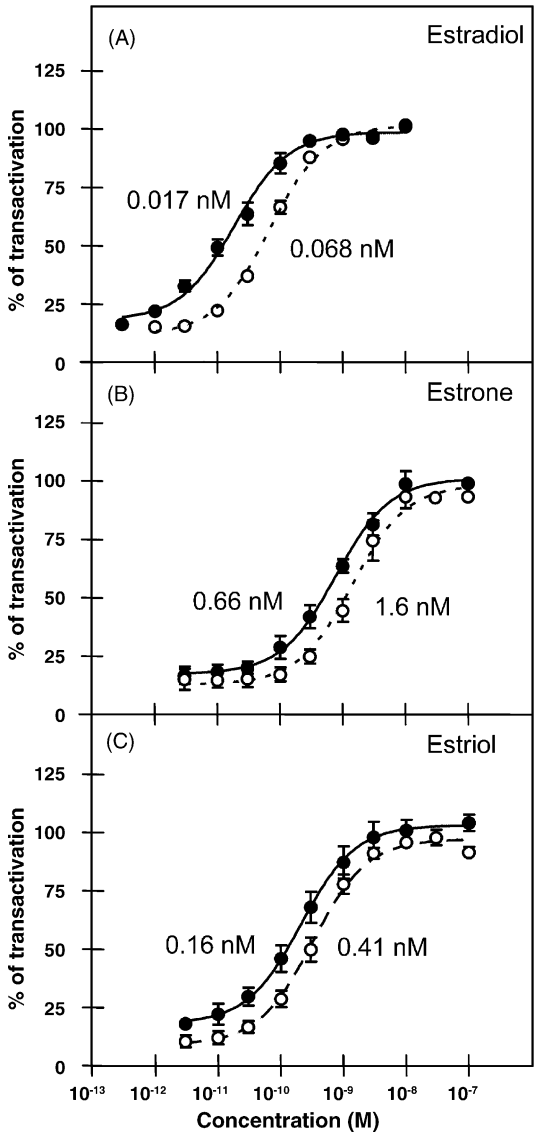

Fig. 2 – Transcriptional activity of ERα and ERβ in response to natural estrogens. HELN-ERα (●) and HELN-ERβ (○) cell lines were treated with to E2 (A), E1 (B), and E3 (C) at the indicated concentrations for 16 h. Maximal activity (100%) corresponds to the activity obtained with 10 nM E2. Values are mean ± S.D. from three separate experiments.

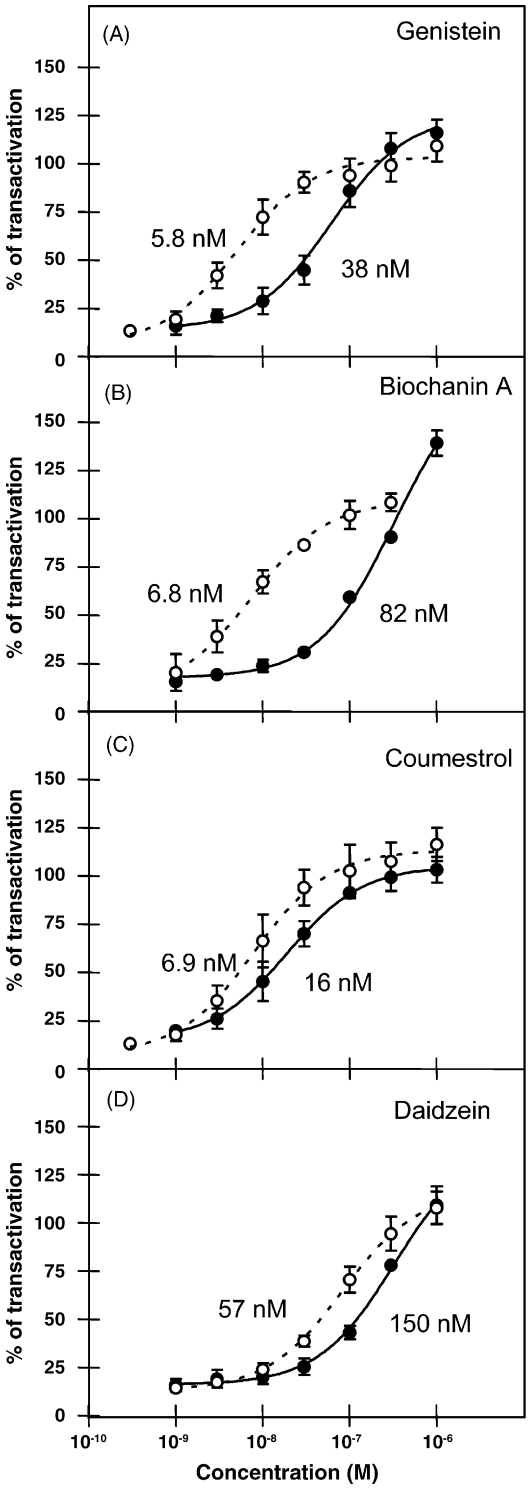

Fig. 3 – Effect of phytoestrogens on ERα and ERβ transcriptional activity. HELN-ERα (●) and HELN-ERβ (○) cell lines were treated with genistein (A), biochanin A (B), coumestrol (C), daidzein (D) at indicated concentrations for 16 h. Results are expressed as indicated in the legend of Fig. 2.

through ERα and ERβ (Fig. 4). EE2 displayed the largest ERα selectivity with a 31-fold higher potency to transactivate luciferase gene expression in HELN-ERα compared to HELN-ERβ cells (EC50 values of 0.008 and 0.25 nM for ERα and ERβ, respectively). Among the 3β,5α-tetrahydro derivatives, 3β,5α-NET showed the best estrogenic potency for ERα (EC50 value of 1.3 nM, compared to 5.6 nM for 3β,5α-GSD and 15 nM for 3β,5α-LNG) but 3β,5α-GSD exhibited the best ERα selectivity (732-fold higher potency to transactivate luciferase gene expression in HELN-ERα cell line – EC50 values of 5.6 nM – compared to 4100 nM in HELN-ERβ cell line). The EC50 value of 3β,5α-NET was very low for ERβ (110 nM) and 3β,5α-GSD, 3β,5α-LNG weakly activated transcription through ERβ. The 3β,5α-GSD

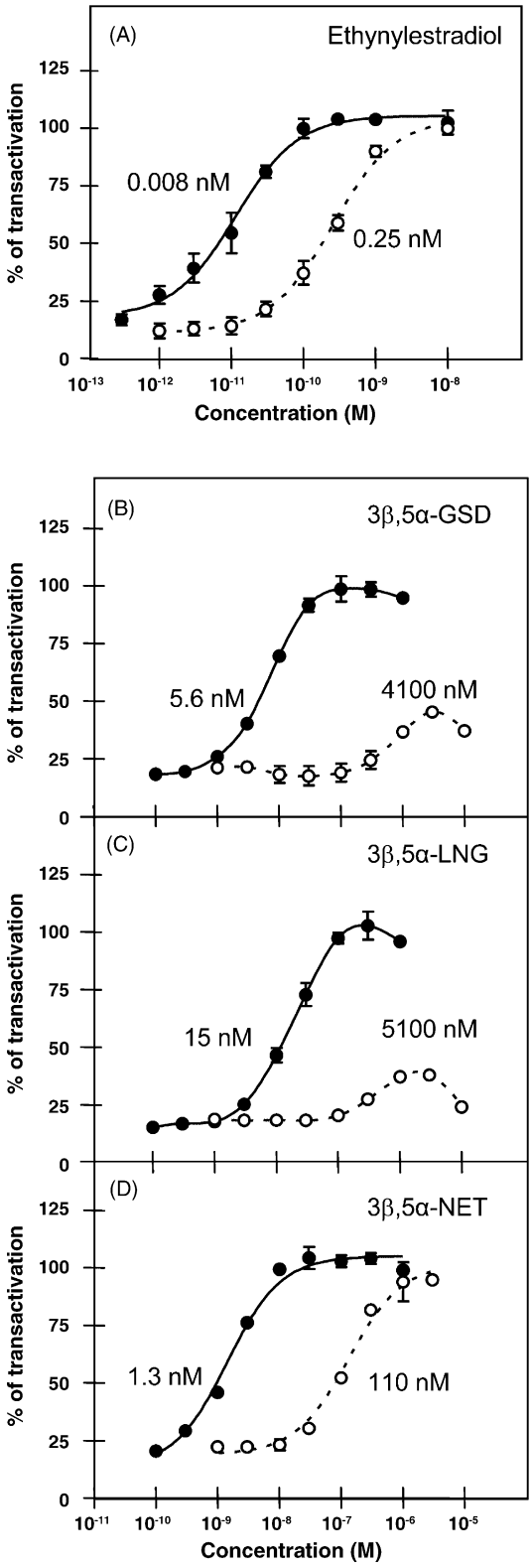

Fig. 4 – Preferential activation of ERα by synthetic steroid derivative ligands. HELN-ERα (●) and HELN-ERβ (○) cell lines were treated with EE2 (A), 3β,5α-GSD (B), 3β,5α-LNG (C), 3β,5α-NET (D) at indicated concentrations for 16 h. Transcriptional activities of ERs were quantified and expressed as indicated in legend of Fig. 2.

and 3β,5α-LNG effects that decreased reporter activity at high concentration were due to cytotoxicity (data not shown).

As shown in Fig. 5, among non-steroidal compounds, PPT showed receptor selective efficacy with a strong potency in transactivating reporter gene expression in HELN-ERα cell line (EC50 value of 0.085 nM). At the opposite, it did not

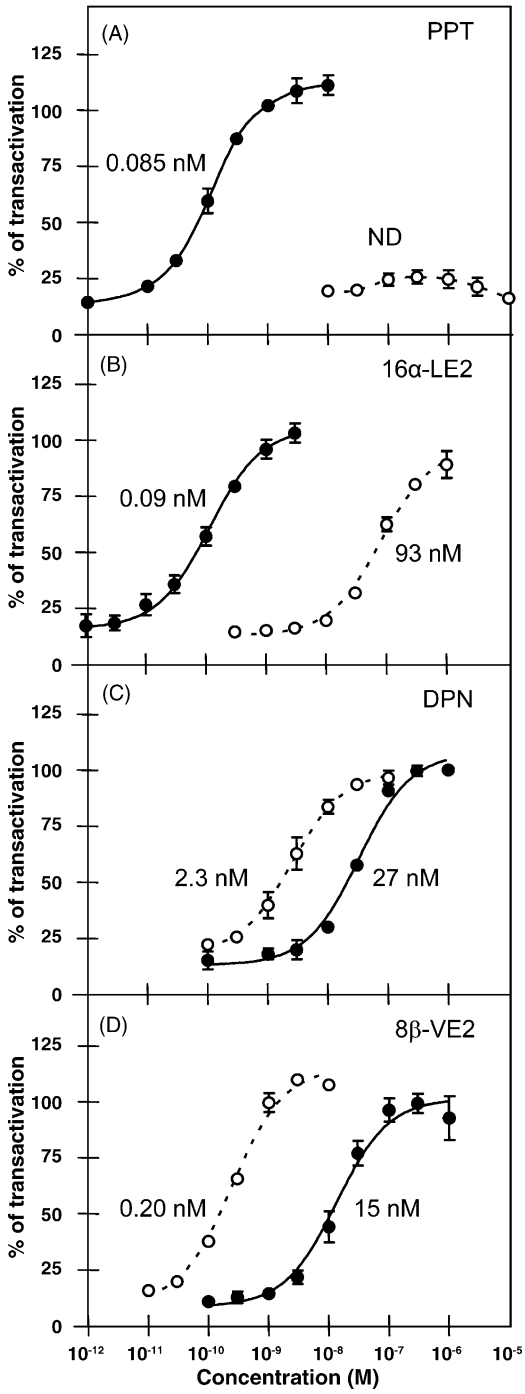

Fig. 5 – Effect of ERα or ERβ selective agonist ligands. Transcriptional activities of ERα (●) and ERβ (○) in response to PPT (A), 16α-LE2(B), DPN (C), and 8β-VE2 (D). HELN-ERs cells lines were treated with compound at the concentrations indicated for 16 h. Results are expressed as indicated in the legend of Fig. 2.

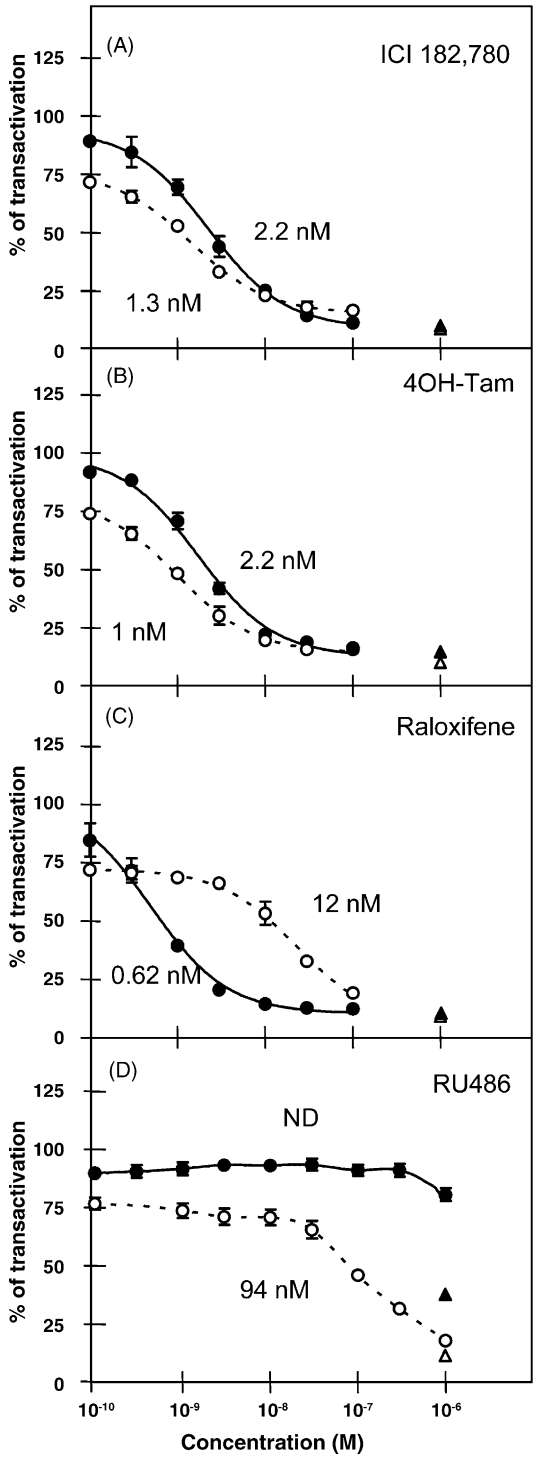

Fig. 6 – Transcriptional response of ERα and ERβ to antiestrogens. The transcriptional response to ICI 182,780 (A), 4OH-Tam (B), raloxifene (C), RU486 (D) in the presence of 0.1 nM E2 was assessed in HELN-ERα (●) and HELN-ERβ (○) cells. Agonistic response to each antiestrogens at 1 μM was also quantified in HELN-ERα (▲) and HELN-ERβ (Δ) cells. Cells were treated with compounds at the indicated concentrations for 24 h. Values are mean ± S.D. from three separate experiments and are expressed as percent of luciferase activity obtained with 10 nM E2.

activate luciferase gene expression in HELN-ERβ cell line (Fig. 5) and furthermore it was a full E2 antagonist (data not shown). DPN, another non-steroidal compound, exhibit EC50 values of 27 nM for ERα and 2.3 nM for ERβ that conferred a 12-fold higher relative potency in transactivation assays for HELN-ERβ cells. Likewise, we tested two isoform selective ligands, which are estradiol derivatives. 16α-LE2 has a 1033-fold selectivity in reporter gene activation via ERα (EC50 values of 0.09 nM for ERα compare to 93 nM for ERβ), and 8β-VE2 displayed a 75-fold ERβ selectivity (EC50 value of 15 nM for ERα compared to EC50 of 0.2 nM for ERβ).

3.5. *Selective transcriptional effects of antagonistic ligands*

The antiestrogenic compounds 4OH-Tam, ICI 182,780, raloxifene and RU486 were tested in HELN-ERα and HELN-ERβ cell lines (Fig. 6) and displayed antagonistic activity. ICI 182,780 and 4OH-Tam potencies were very similar towards the two reporter cell lines (1.7- and 2.2-fold more potent estrogen antagonists for ERβ than ERα, respectively). By sharp contrast, raloxifene was a 19-fold more potent estrogen antagonist for ERα (IC50 value of 0.62 nM) than for ERβ (IC50 value of 12 nM) whereas RU486 was an antagonist for ERβ (IC50 value of 94 nM). In parallel, we tested the agonistic activity of the four antiestrogens at 1 μM. As shown in Fig. 6, they were all devoid of partial agonist activity in HELN-ERβ cells, whereas 4OH-Tam and RU486 induced luciferase activity in HELN-ERα cell lines (15 and 37%, respectively).

3.6. *Selective activation of ERβ by combining agonistic and antagonistic ligands*

Because all the ERβ selective compounds tested were also able to activate ERα, we associated ERβ agonists with the ERα selective antagonist raloxifene. Dose-titrations of genistein in presence of fixed concentrations of raloxifene (1–100 nM) showed that low concentrations of raloxifene caused a rightward shift in the EC50 value for genistein in HELN-ERα cell compared to HELN-ERβ cells (Fig. 7). In the presence of 10 nM raloxifene, 300 nM genistein almost fully activated ERβ without increasing ERα transactivation. Association of agonistic and antagonistic ligands therefore strongly improved ERβ selectivity thus permitting to specifically activate this receptor subtype.

4. Discussion

Estrogens regulate gene transcription through binding to nuclear estrogen receptors (ERα and ERβ). Although they share a high sequence similarity, the two ER subtypes differentially regulate target gene transcription and play specific physiopathological roles. There is therefore a real interest in identifying molecules able to specifically activate or inhibit ER subtypes.

To analyze specificity in term of binding and transcriptional activation of ERα and ERβ, we used reporter cell lines expressing human ERα or ERβ that we previously established in our laboratory. Since these cell lines were constructed from the same intermediary HELN cells (derived from HeLa

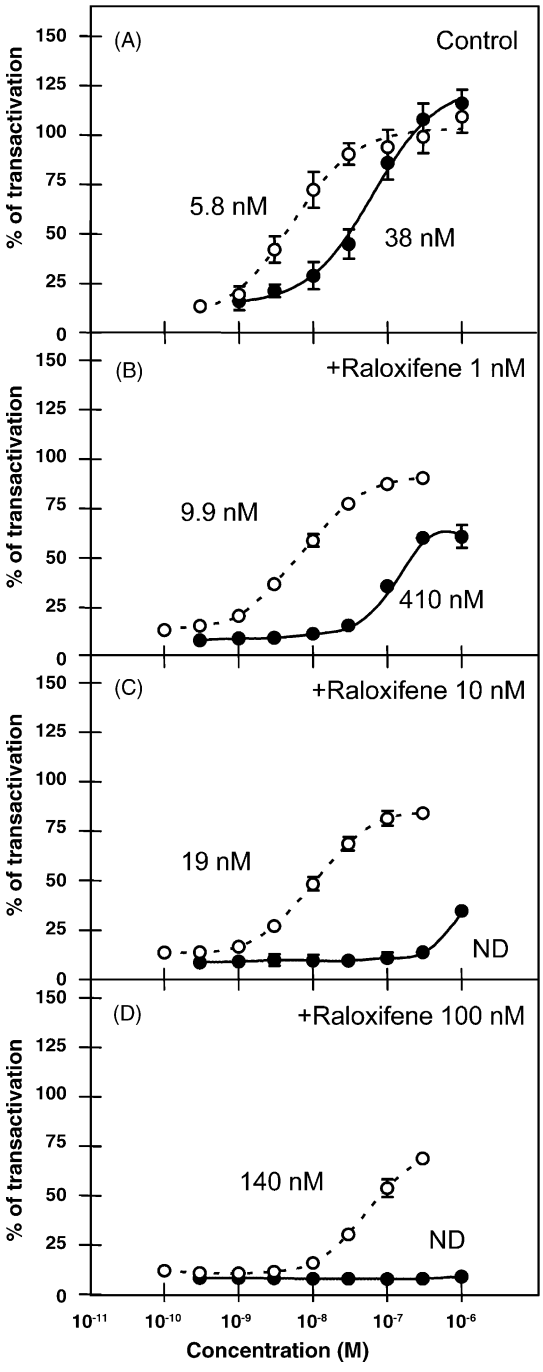

Fig. 7 – Specific activation of ERβ by a combination of agonist and antagonist ligands. HELN-ERα (●) and HELN-ERβ (○) cells were exposed for 24 h to increasing concentrations of genistein in the absence (control) or in the presence of 1, 10 or 100 nM raloxifene. Values are mean ± S.D. from three separate experiments and are expressed as percent of luciferase activity obtained with 10 nM E2.

cells), we could use them as powerful tools to compare the transcriptional responses of ERα and ERβ to various estrogen agonists and antagonists.

Our data show that most of the tested compounds presented specific responses, although some ligands gave similar responses for ERα and ERβ. Interestingly, results obtained in transactivation experiments were strongly corroborated by ligand affinity values determined using whole-cell ligand-binding assays. Crystallography studies of ERα and ERβ ligand-binding domains (LBD) have revealed that amino acid residues that line the surface of the ligand-binding cavity are localized from helix3 to helix12 [23]. Note that these residues are extremely conserved in ERα and in ERβ. In fact, among the 22–24 residues that are considered to be in contact with the ligand (i.e., within 4 Å) all but two are identical in ERα and ERβ. These are ERα-Leu 384 corresponding to ERβ-Met 336 and ERα-Met 421 corresponding to ERβ-Ile 373. The role of Leu 384 and Met 336 (respectively in ERα and ERβ) was investigated by mutagenesis demonstrating the importance of this position either in transactivation [24] or binding [25] selectivity.

Altogether, the results obtained with our HELN-ER cells were in agreement with those of the literature. For instance, we confirmed that PPT and 16α-LE2 were strong selective ligand agonist for ERα [26]. For PPT, the selectivity depends on particular interactions between its pyrazole core and the C4-propyl groups with a region on the ligand-binding pocket where ERα has a smaller residue (Leu 384) than ERβ (Met 336) [26]. In 16α-LE2, the larger substitution in position 16α displaces the flexible methionine (Met 421), thus introducing an unfavorable interaction with the rigid isoleucine (Ile 373) [21]. Among the 3β,5α-tetrahydrometabolites, 3β,5α-NET showed the best estrogenic potency for ERα, but 3β,5α-GSD had the best ERα selective efficacy.

Among all the synthetic ligands tested, DPN and 8β-VE2 were the best ERβ selective ligand with 8β-VE2 showing the highest ERβ potency and selectivity. For DPN, selectivity is due to one residue (Met 336) in the ERβ LBD, which appears to interact with the DPN nitrile function and to some differences in helix3 which reshape the ligand-binding pocket, improving productive interactions between receptor and this rather small ligand [25]. For 8β-VE2, the larger substitution at position 8β increases ERβ selectivity [21]. We showed that biochanin A and genistein, in addition to DPN and 8β-VE2, were also selective for ERβ. The relative order of magnitude of biochanin A and genistein ligand-binding affinity was smaller in our work than in a previous study [27]. Again, it is important to note that our assay was performed in whole cells whereas the study by Kuiper et al. was realized in vitro. Biotransformation undergoing in living cells might turn some ligands, like biochanin A, into less potent metabolites and explain the difference between the two methods [28].

A reduced number of compounds have been described to be selective antagonists. MPP (methyl-piperidino-pyrazole) has no stimulatory activity on ERα or ERβ, whereas it specifically and fully inhibits ERα activity by E2 [29]. As reported by others, *R,R*-THC (*R,R*-tetrahydrochrysene) appears to be an agonist on ERα and a full antagonist on ERβ [30]. Finally, the *R* enantiomer indenestrol fully transactivates ERβ but acts only as a partial agonist on ERα [24].

In our study, the affinity of antagonists was slightly different from that in studies where binding experiments were performed with receptors either synthesized in vitro using reticulocyte lysates or obtained from insect cell extracts [19,27]. In intact cells, ligand-binding affinities could be modified by ER associated proteins [31], thus explaining the

difference of RBA obtained in binding assays performed on cell extracts or with purified ERs. In our cell lines, we saw that raloxifene was the most selective antagonist for ERα and RU486 the most selective antagonist for ERβ. Raloxifene exhibited an ERα-selective partial agonist/antagonist function but a pure antagonistic effect through ERβ which was also shown by others [22]. RU486, which is a 19-nortestosterone derivative and a potent antiprogestin, displayed an ERβ selective antagonistic activity. This is related to its ability to bind ERβ as shown in whole-cell assays (Table 1) [32]. By contrast, we found that RU486 acts as partial agonist for ERα as previously demonstrated [33].

In the case of ERα, the N-terminal AF-1, through its interaction with coactivators or corepressors, appears critical for the mixed agonistic/antagonistic transcriptional properties of SERMs [34]. Interestingly, phytoestrogens could be regarded as natural SERMs for ERβ. For instance, crystallography data obtained in the absence of a coactivator fragment, showed a structure exhibiting partial agonist conformation of helix12 [35]. The partial agonistic activity of genistein on ERβ was also reported in embryonic kidney cells with a shorter form of ERβ [22]. Future studies will be necessary to assess the potential role of AF-1 in the ERβ-mediated agonist activity of phytoestrogens.

In the present paper, we correlated the selectivity of ligands for binding to the two ERs with their ability to regulate ERE-mediated transactivation by the two subtypes. It would obviously be interesting to investigate other parameters such as ERE-independent transactivation through Sp1 or AP-1 transcription factors. In addition, the selectivity should also be validated on more general parameters such as cell proliferation.

Finally, the last observation of the present work deals with selective activation of ERβ. Until now, the selectivity of ERβ agonists has been lower than that of ERα agonists. Indeed, none of the ligands tested appeared totally selective for ERβ whereas, on the other hand, PPT showed full stimulation through ERα without displaying any agonistic activity through ERβ. Very interestingly, the association of an ERα antagonist (raloxifene) with an ERβ agonist such as genistein (Fig. 7), 8β-VE2 or DPN (data not shown) enabled us to obtain a transcriptional response for ERβ only. Therefore, even when both receptors are present in the same cell, treatment with PPT or with the combination of raloxifene plus an ERβ selective agonist offers the interesting possibility to study and specifically activate one of the two ER homodimers or one partner of an ERα/ERβ heterodimer [36]. This observation also appears of particular relevance in physiopathology since the association of raloxifene with phytoestrogens actually occurs in patients who receive raloxifene as a preventive therapy of postmenopausal osteoporosis [37] and have phytoestrogens in their diet. Further studies will be necessary to evaluate a long-term use of treatments associating raloxifene with phytoestrogens.

In conclusion, it is clear that the design or identification of new ER-selective ligands will drive further research. Our HELN-ER cells, with their specific estrogen-responsive reporter genes, will certainly be useful tools for the characterization of such molecules, which should be valuable (alone or in combination) for the treatment of estrogen-responsive diseases.

Acknowledgements

The authors are grateful to Dr. H Gronemeyer and Pr. P Chambon for the gift of plasmids. We are also extremely grateful to Dr. J.L. Borgna and Dr. M.J. Duchesne for comments on the manuscript. This study has been partly supported by the European Union network CASCADE (FOOD-CT-2003-506319).

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