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Rapid yeast estrogen bioassays stably expressing human estrogen receptors α and β, and green fluorescent protein: a comparison of different compounds with both receptor types

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Abstract

Previously, we described the construction of a rapid yeast bioassay stably expressing human estrogen receptor α (hERα) and yeast enhanced green fluorescent protein (yEGFP) in response to estrogens. In the present study, the properties of this assay were further studied by testing a series of estrogenic compounds. Furthermore, a similar assay was developed based on the stable expression of human estrogen receptor β (hERβ). When exposed to 17β-estradiol, the maximum transcriptional activity of the ERβ cytosensor was only about 40% of the activity observed with ERα, but the concentration where half-maximal activation is reached (EC_{50}), was about five times lower. The relative estrogenic potencies (REP), defined as the ratio between the EC_{50} of 17β-estradiol and the EC_{50} of the compound, of the synthetic hormones dienestrol, hexestrol and especially mestranol were higher with ERα, while DES was slightly more potent with ERβ. The gestagens progesterone and medroxyprogesterone-acetate showed no response, whereas the androgen testosterone showed a very weak response. The anabolic agent, 19-nortestosterone showed a clear dose-related response with estrogen receptor α but not β. The phytoestrogens coumestrol, genistein, genistin, daidzein, daidzin and naringenin were relatively more potent with ERβ. Ranking of the estrogenic potency with ERα was: 17β-estradiol ≫ 8-prenylnaringenin > coumestrol > zearalenone ≫ genistein ≫ genistin > naringenin. The ranking with the ERβ was: 17β-estradiol ≫ coumestrol > genistein > zearalenone > 8-prenylnaringen ≫ daidzein > naringenin > genistin ≫ daidzin. The hop estrogen 8-prenylnaringenin is relatively more potent with ERα. These data show that the newly developed bioassays are valuable tools for the rapid and high-throughput screening for estrogenic activity.

Keywords: Estrogen receptor alpha; Estrogen receptor beta; Yeast bioassay; 8-prenylnaringenin

1. Introduction

There is serious concern that chemicals in our food, water and environment may affect human health by disrupting normal endocrine function. This relates both to certain chemicals with previously unknown hormonal properties, and compounds used, e.g. for their growth-promoting properties in animals. On the other hand, the estrogenic properties of the soy isoflavones genistein and daidzein are thought to play a role in their putative health-enhancing properties, such as prevention of certain cancers [1], decreased risk of cardiovascular diseases [2], and the improvement of bone health [3]. Soy isoflavones have also been reported to prevent growth of breast cancer cells [4,5].

An important role in this apparent controversy may be played by the two estrogen receptors (ER) that have been identified to date [6,7]. These receptors, derived from two different genes and referred to as ERα and ERβ, both bind to the consensus estrogen responsive element (ERE) enabling the transcription of an ERE-based reporter construct. The DNA-binding domains of ERα and ERβ show a high degree of homology but the ligand-binding domains show only 59% homology. In general, both receptors display similar ligand-binding profiles, but phytoestrogens like genistein and coumestrol induce receptor-dependent transcription stronger with ERβ than with ERα [8,9]. Furthermore, the tissue distribution of ERα and ERβ also differs substantially [8,10]. ERβ is very important in the bone, urogenital tract,

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cardiovascular system, central nervous system and the developing brain [11,12]. ERα seems the more important receptor type in the mammary gland and the uterus. It has been hypothesised that ERβ is important for the protection against hyperproliferation and carcinogenesis in breast, prostate and the gastrointestinal tract [11,13–18]. These observations led to the hypothesis of potential differences in the biological function and tissue-selective actions of the two receptors. These observations also suggest the existence of two previously unrecognised pathways of estrogen signalling: via the ERβ subtype in tissues exclusively expressing this subtype and, since estrogen receptors are known to function as dimers [19], via the formation of heterodimers in tissues expressing both subtypes. The differences in tissue distribution may also be very important from a pharmaceutical point of view, as hormone replacement therapy in postmenopausal women is an increasingly significant health issue [20].

In order to investigate the properties of chemicals and the presence of hormonally active substances in food and water, it is important to have rapid, robust and high-throughput bioassays. The effects of estrogens on different tissues stress the need for bioassays with both estrogen receptor types. When sensitivity is required, like in cases of small sample volumes and/or low concentrations, mammalian assays can best be used as they display lower detection limits than yeast-based assays [21]. However, because of the differences observed in the ERα and ERβ activity in different cell lines [22,23], the differences in estrogenic activities of substances by both receptor types can as well be studied in yeast estrogen transcription activation assays with a consensus ERE-reporter construct. Furthermore, toxicity of samples to yeast or mammalian cells is a potential problem in assessing estrogenic activity in complex samples. As cytotoxicity occurs more frequently in mammalian cell assays than in yeast assays [24,25] and because yeast is more resistant to environmental contaminants, such as heavy metals and bacterial endotoxins [26], yeast assays can best be used to study the estrogenic activity in such samples.

Several different yeast assays have been described, all of them using plasmid-based constructs for either the receptor or reporter construct, and β-galactosidase as the reporter protein [9,27–29]. With the exception of Morito et al. [9] these assays only use the human estrogen receptor α (hERα). Previously, we described the construction of a rapid yeast bioassay, with constructs for the human estrogen receptor α and the ERE-reporter construct stably integrated into the genome [30]. This ERE-reporter construct consists of two consensus EREs with a centre-to-centre spacing of 40 bp that was placed in a truncated CYC1 promoter. Furthermore, yeast enhanced green fluorescent protein (yEGFP) was used as reporter protein, allowing a much easier and more rapid detection of estrogenic activity. In the present study, we describe the development and properties of a similar assay based on the stable expression of human estrogen receptor β (hERβ). In order to validate both assays, a large number of compounds with known estrogenic properties were tested. These include natural hormones and their conjugated forms and metabolites, synthetic hormones like ethynylestradiol, estradiolbenzoate and DES, phytoestrogens like genistein, coumestrol and daidzein, the mycotoxin zearalenone and its derivatives, and environmental pollutants like *p*-nonylphenol.

2. Materials and methods

2.1. Chemicals

The following compounds were purchased from Sigma: 17β-estradiol (E2β, CASRN 50-28-2), 17α-estradiol (E2α, 57-91-0), 17β-estradiol 3-benzoate (E2-benz, 50-50-0), 17β-estradiol 3-sulfate (E2-sul, 4999-79-5), 17β-estradiol 3-(β-D-glucuronide) (E2-3-gluc; 14982-12-8), 17β-estradiol 17-(β-D-glucuronide) (E2-17-gluc, 15087-02-2), estrone (E1, 53-16-7), estrone 3-sulfate (E1-sul, 438-67-5), estrone β-D-glucuronide (E1-gluc, 15087-01-1), estriol (E3, 50-27-1), 17α-ethynylestradiol (EE2, 57-63-6), dienestrol (84-17-3), mestranol (72-33-3), medroxyprogesterone 17-acetate (MPA, 71-58-9), progesterone (P, 57-83-0), testosterone (T, 58-22-0), 19-nortestosterone (19-norT, 434-22-0), 2-deoxy-20-hydroxyecdysone (β-ecdysone, 17942-08-4), zearalenone (z-lenone, 17924-92-4), zearalanone (z-lanone, 5975-78-0), α-zearalenol (α-z-lenol, 36455-72-8), α-zearalanol (α-z-lanol, 26538-44-3), β-zearalenol (β-z-lenol, 71030-11-0), β-zearalanol (β-z-lanol, 42422-68-4), genistein (446-72-0), genistin (529-59-9) and daidzein (486-66-8). Daidzin (552-66-9) was obtained from Plantech (UK), diethylstilbestrol (DES, 56-53-1) from Interpharm (The Netherlands) and hexestrol (84-16-2) from ICN. Coumestrol (479-13-0), enterolactone (78473-71-9), enterodiol (80226-00-2) and 4-nonylphenol (NP, 84852-15-3/104-40-5) were obtained from Fluka and 4-*n*-nonylphenol (4nNP, 104-40-5) from Riedel-de Haën. Naringenin (Nar, 480-41-1) and 8-prenylnaringenin (8-prenylN) were purchased from Apin Chemical Limited (UK) and 2-hydroxyestradiol (2OH-E2, 362-05-0), 4-hydroxyestradiol (4OH-E2, 5976-61-4), 2-hydroxyestrone (2-OH-E1, 362-06-1) and 4-hydroxyestrone (4-OH-E1, 3131-23-5) were obtained from Steraloids (USA). From all these compounds fresh stock solutions were made in either ethanol or DMSO as indicated.

2.2. Yeast strain

The yeast *Saccharomyces cerevisiae* (CEN.PK 102-5B, K20, URA3$^-$, HIS3$^-$, LEU$^-$) was a gift from H. Silljé (University of Utrecht).

2.3. Plasmids

For the expression of the human estrogen receptor α, the p403-GPD yeast expression vector was used. For the expression of the human estrogen receptor β, the p405-

GPD yeast expression vector was used. For the construction of the reporter plasmid, the p406-CYC1 yeast expression vector was used. All three plasmids were obtained from the American Type Culture Collection (ATCC, Rockville, Maryland, USA) and are described by Mumberg et al. [31].

2.4. Construction of the p405-GPD-ERβ receptor expression vector

The construction of the p403-GPD-ERα receptor expression vector, that contains the HIS3 marker gene, is already described in Bovee et al. [30]. The p405-GPD-ERβ receptor expression vector, containing the LEU marker gene, is made in a similar way. Briefly, synthesis of cDNA was carried out on isolated mRNA of T47D human breast cancer cells and of mRNA isolated from human intestinal Caco-2 cells. Full-length human estrogen receptor β cDNA was obtained by PCRs using the T47D cDNA, marathon uterus cDNA (human, Clontech) and the human intestine cDNA with the Expand High Fidelity PCR System (Boehringer Mannheim). PCR was performed using the following conditions: 34.2 μl ultra pure water, 5 μl 25 mM $MgCl_2$, 5 μl Expand HF 10× concentrated buffer (without $MgCl_2$), 0.8 μl 25 mM dNTP mix, 1 μl of the enzyme mix, 2 μl of the different cDNAs and 2 μl of a primer mix containing 10 μM of each primer were pipetted into a thin-walled PCR tube and PCR was performed in an Eppendorf Mastercycler gradient using the following cycle profile: (1) denature template 3 min at 95 °C; (2) denature template 30 s at 94 °C; (3) anneal primers 1 min at 60 °C; (4) elongation 2 min at 72 °C; (5) go to step (2) and repeat 35 times; (6) elongation 7 min at 72 °C; and (7) for over 10 °C. After this first PCR, a second PCR was performed with the same conditions as described above but now 2 μl of the first PCR mixture was used instead of 2 μl of the different cDNAs. The sequence of the 5′-primer was as follows: 5′-CGTCTAGAGCTGTTATCTCAAGACATGGATATAA-3′ and this primer contains a restriction site for *Xba*I just before the ATG start codon. The sequence of the 3′-primer is as follows: 5′-TAGGATCCGTCACTGAGACTGTGGGTTCTG-3′ and this primer contains a restriction site for *Bam*HI just after the TGA stop codon. This PCR generated a full-length ds cDNA of 1626 bp containing the 1593 bp coding sequence of the human estrogen receptor β gene with a 5′-*Xba*I and a 3′-*Bam*HI restriction site just outside the coding sequence. This full-length ERβ PCR product was isolated from a 1% low-melt agarose gel and ligated into a pGEM-T Easy Vector (Promega). Plasmid digestion control revealed several good clones and good clones were sequenced in both directions using the SEQ 4×4 apparatus and the Thermo Sequenase Cy5.5 dye terminator cycle sequencing kit, all used according to the manufacturers instructions (Amersham Pharmacia). All 1593 bp, from the ATG start to the TGA stop, were compared with the estrogen receptor β sequence published by Ogawa et al. [32].

Compared to this sequence, the ERβ cDNA clone obtained from the breast cells contained two mutations. The bp at position #430 consisted of T instead of A and #1030 consisted of G instead of A. Both mutations resulted in changed codons that will change the amino acid translated from it, Arginine to Tryptophan and from Methionine into Valine, respectively. Probably this clone also misses a G at bp position #216. This clone was therefore not used. The ERβ cDNA clone obtained from human uterus contained five mutations. The bp at position #349 consisted of C instead of T, #601 C instead of T, #800 G instead of A, #965 C instead of T and #1245 consisted of G instead of A. The mutations at bp positions #349 and #1245 result in changed codons, but these changed codons will be translated into the same amino acids as the original codons. However, the mutations at bp positions #601, #800 and #965 result in changed codons that also change the amino acid that will be translated from it, Cysteine to Arginine, Glutamine to Arginine and from Leucine to Proline, respectively. This clone was therefore not used. The ERβ cDNA clone obtained from human intestine contained 7 A nucleotides instead of 6 A nucleotides at bp position #9 to #14. This will result in a frame shift that changes the entire sequence and therefore this clone was not used.

To obtain a cDNA clone that is fully complementary to the sequence published by Ogawa et al. [32], the *Xba*I–*Msc*I part from this intestine ERβ cDNA clone, containing the 7 A instead of 6 A, was replaced by the *Xba*I–*Msc*I part of the uterus ERβ cDNA clone. Although the uterus ERβ cDNA clone contained five mutations, the first mistake is at bp position #349 and so the *Xba*I–*Msc*I part of this clone does not contain any mistake. In this way a human ERβ cDNA clone was obtained that fully corresponded to the estrogen receptor β sequence published by Ogawa et al. [32]. This uterus/intestine human ERβ cDNA was cut out of the pGEM-T Easy plasmid with *Xba*I and *Bam*HI and cloned into the corresponding *Xba*I–*Bam*HI site of the p405-GPD expression vector. This p405-GPD-ERβ vector was used to transform Epicurian Coli XL-2 Blue Cells. Plasmid digestion controls and PCR controls of single white colonies were performed and revealed several good clones (data not shown).

2.5. Construction of yeast hERα and hERβ cytosensors

The yeast cytosensor expressing the hERα is the one already described in Bovee et al. [30]. The yeast cytosensor expressing the hERβ and the yeast cytosensor that expresses both ERα and ERβ are made in a similar way. Briefly, construction of yeast hERα and hERβ cytosensors was started with the stable transformation of yeast K20 (Ura^-, His^- and Leu^-) with the p406-ERE_2s2-CYC1-yEGFP reporter vector, integrated at the chromosomal location of the Uracil gene via homologous recombination. Transformants were grown on MM/LH plates and PCR and Southern blot hybridisation were used to select clones in which the integration has occurred at the desired URA3 site with only a

single copy of this reporter vector. Subsequently, this yeast reporter strain was stably transformed with the p403-GPD-ERα, the p405-GPD-ERβ or both expression vectors and transformants were grown, respectively on MM/L, MM/H or MM plates. Actually, the cytosensor containing both receptor types was made by transforming the ERα cytosensor with the p405-GPD-ERβ receptor expression vector.

2.6. Yeast culturing conditions

Before running an assay, an agar plate containing the selective MM/L, MM/H or MM medium was inoculated with the yeast ERα, ERβ or ERα/β cytosensor, respectively from a frozen −80 °C stock (20% glycerol, v/v). The plate was incubated at 30 °C for 24–48 h and then stored at 4 °C. The day before running the assay, a single colony of the yeast cytosensor was used to inoculate 10 ml of the corresponding selective medium. This culture was grown overnight at 30 °C with vigorous orbital shaking at 225 rpm. At the late log phase the yeast ERα cytosensor culture was diluted (1:10) in MM/L, the yeast ERβ cytosensor was diluted (1:20) in MM/H and the yeast ERα/β cytosensor was diluted (1:20) in MM. This minimal medium (MM) consisted of yeast nitrogen base without amino acids or ammonium sulphate (1.7 g/l), dextrose (20 g/l) and ammonium sulphate (5 g/l). The MM/L and MM/H medium were supplemented with L-leucine (60 mg/l) or L-histidine (2 mg/l), respectively.

2.7. yEGFP assay: exposure to different substances and the measurement of estrogenic activities

For exposure in 96 well plates (Costar), aliquots of 200 μl of the yeast culture were pipetted into each well. Exposures to different doses of 17β-estradiol and all the other substances were performed through the addition of 1 μl of an ethanol or DMSO stock solution to each well, resulting in 0.5% final concentration of the solvent. Ethanol and DMSO only controls were included in each experiment and each sample concentration was assayed in triplicate. Exposures were performed for 4 and 24 h. Fluorescence at these time intervals was measured directly in the CytoFluor Multi-Well Plate Reader (Series 4000, PerSeptive Biosystems) using excitation at 485 nm and measuring emission at 530 nm. The densities of the yeast culture at these time intervals were also determined by measuring the OD at 630 nm. This was done to check whether a substance was toxic for yeast. If there were no differences in yeast densities, the fluorescence signals were corrected with the signals obtained with the blank medium (supplemented MM containing 0.5% ethanol or DMSO solvent only).

3. Results

Recombinant yeast cells were constructed that either express the human estrogen receptor α, β or both, and yEGFP in response to exposure to estrogens. All constructs, both the receptor construct as well as the reporter constructs, were stably integrated into the yeast genome by the use of yeast integrating plasmids. The construction of the yeast cell expressing the hERα was described previously, including that of the cDNA encoding for the alpha receptor [30]. This cDNA, constructed from mRNA of T47D cells, had the same sequence as described by Greene et al. [33]. The construction of cDNA for the hERβ-receptor was initially attempted from the mRNAs isolated from T47D human breast cancer cells, human intestinal Caco-2 cells and from human uterus cDNA. Since all three cDNAs contained mutations in comparison to the sequence published by Ogawa et al. [32], the eventual cDNA introduced into the yeast cytosensor was constructed from the latter two cDNAs (see Section 2.4).

A number of compounds with known estrogenic properties were tested in the ERα-assay. As shown in Fig. 1, compounds like DES, ethynylestradiol, and genistein all caused a dose-related increase in the production of green fluorescent protein after a relatively short exposure period of 4 h. Dose–response curves were similar after 24 h but in general allowed a better curve-fit (Fig. 2A and 2B).

Fig. 3 shows the dose–response curve for 17β-estradiol obtained after 4 h with the yeast cytosensors expressing hERα, hERβ or both the hERα and hERβ receptors. The maximal transcriptional activity of the ERβ cytosensor is only about 40% of the maximal activity observed with the ERα cytosensor, but the ERβ cytosensor showed a higher response at lower concentrations. This is reflected in the much lower EC_{50} for 17β-estradiol, the concentration where half-maximal activation is reached, being 0.06 and 0.6 nM, respectively for the ERβ and ERα cytosensor. In contrast to the ERα cytosensor, the dose–response curves obtained with the ERβ and the ERα/ERβ cytosensors after a 24 h exposure period were very poor (data not shown).

Fig. 4A and 4B show dose–response curves obtained with the ERβ cytosensor after a 4 h exposure to a number of different compounds. Table 1 shows the calculated EC_{50} values for 17β-estradiol, a number of metabolites and other hormonal substances, as obtained by a mathematical non-linear regression curve-fit formula ($y = a0+a1/(1+(x/a2)\text{^}a3)$). The relative estrogenic potencies (REP) of these substances, defined as the ratio between the EC_{50} of 17β-estradiol and the EC_{50} of the compound, with both the ERα and the ERβ cytosensor are also shown in Table 1. Fig. 5 presents a graphical comparison of these EC_{50} values. These data show that, e.g. estrone, the main metabolite of 17β-estradiol, showed a REP of 0.2 with ERα and 0.1 with ERβ. Other metabolites of E2β, like the hydroxy metabolites and the sulfate and glucuronide conjugates, are much less potent than E2β itself, displaying relative potencies of less than 0.05. However, the hydroxy-metabolites are more potent than the conjugated forms. There are only small differences between the relative potencies with ERα and ERβ of these natural hormones and their conjugated forms and metabolites. However, like estrone, the conjugated forms (estrone 3-sulfate and estrone

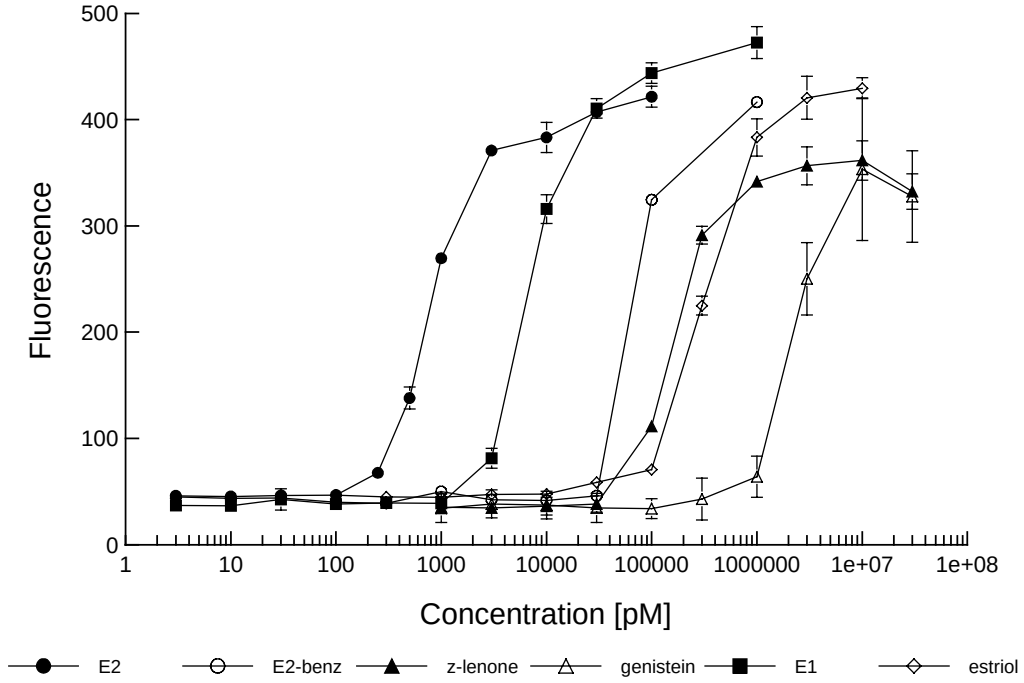

Fig. 1. Response of the yeast ERα cytosensor to different substances after a 4 h exposure period. Exposure to 17β-estradiol and other substances was started by adding to 200 μl of a yeast culture of the ERα cytosensor, an aliquot of 1 μl of a stock solution, using either ethanol or DMSO as a solvent as indicated in Tables 1 and 2. Fluorescence was determined after 4 h as described in Section 2 (see Section 2.7). Cells were exposed to 17β-estradiol (E2), β-estradiol 3-benzoate (E2-benz), zearalenone (z-lenone), genistein, estrone (E1), or estriol. Fluorescence signals are the mean of a triplicate with S.D.

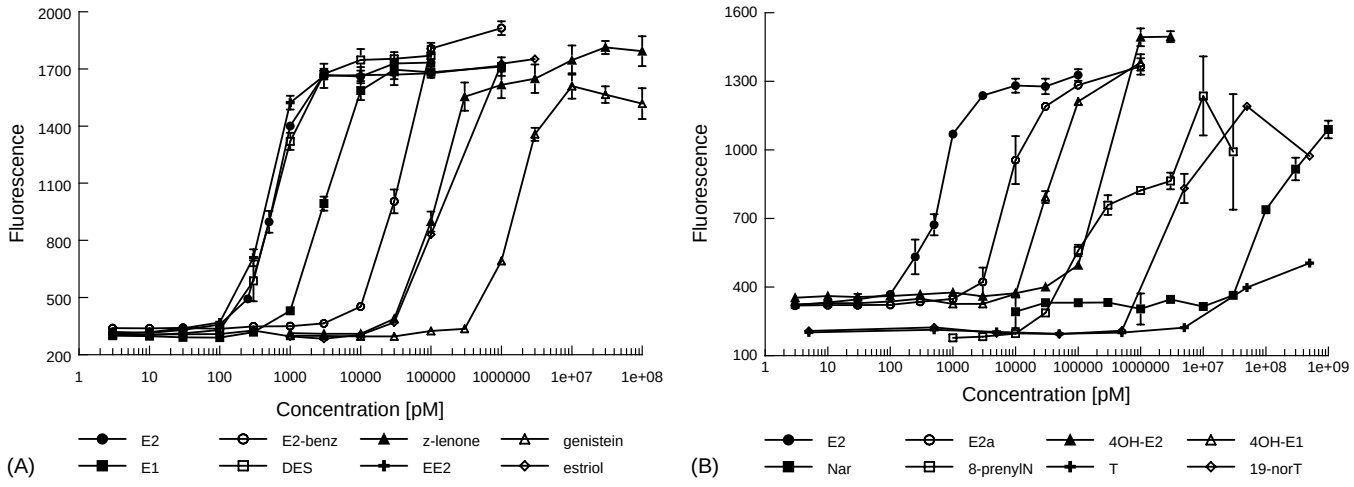

Fig. 2. Response of the yeast ERα cytosensor to different substances after a 24 h exposure period. Exposure to 17β-estradiol and other substances was started by adding to 200 μl of a yeast culture of the ERα cytosensor, an aliquot of 1 μl of a stock solution, using either ethanol or DMSO as a solvent as indicated in Tables 1 and 2. Fluorescence was determined after 24 h as described in Section 2 (see Section 2.7). (A) shows dose–response curves of 17β-estradiol (E2), β-estradiol 3-benzoate (E2-benz), zearalenone (z-lenone), genistein, estrone (E1), diethylstilbestrol (DES), 17α-ethynylestradiol (EE2) and estriol and (B) shows dose–response curves of 17β-estradiol (E2), 17α-estradiol (E2α), 4-hydroxyestradiol (4OH-E2), 4-hydroxyestrone (4OH-E1), naringenin (Nar), 8-prenylnaringenin (8-prenylN), testosterone (T) and 19-nortestosterone (19-norT). Fluorescence signals are the mean of a triplicate with S.D.

β-D-glucuronide) and metabolites (2-hydroxyestrone and 4-hydroxyestrone) are slightly more potent with ERα than they are with ERβ (see Fig. 5). The REP with ERα and ERβ of 2-OH-E1 are 0.0026 and 0.00026, respectively; of 4-OH-E1 0.022 and 0.0048, respectively; of E1-3-sulfate 5×10^{-5} and 1.1×10^{-5}, respectively; and of E1-β-D-glucuronide $<1 \times 10^{-5}$ and 4×10^{-6}, respectively. The differences with the synthetic hormones dienestrol, hexestrol, mestranol and DES are more obvious. Dienestrol, hexestrol and especially mestranol are relatively much more potent with the ERα, showing REPs of 0.56, 0.36 and 0.11, respectively than with the ERβ, showing REPs of 0.091, 0.091 and 0.0001, re-

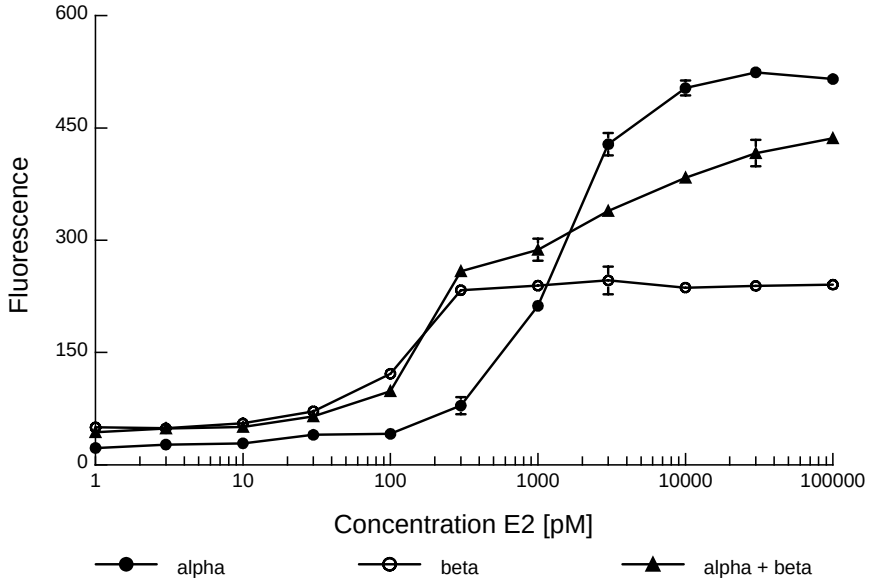

Fig. 3. Response of the yeast ERα cytosensor, ERβ cytosensor and the yeast cytosensor that expresses both the ERα and the ERβ after exposures for 4 h to 17β-estradiol (E2). Exposure was started by adding to 200 μl yeast culture of the corresponding cytosensors, an aliquot of 1 μl of an E2 stock solution in DMSO. Fluorescence was determined after 4 h as described in Section 2 (see Section 2.7) and fluorescence signals are the mean of a triplicate with S.D.

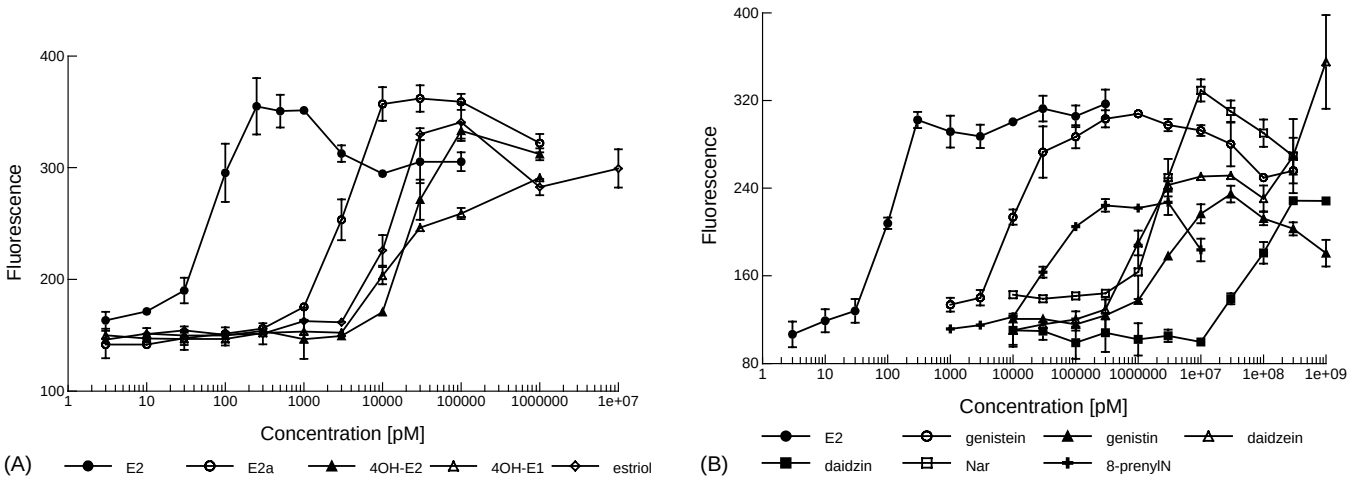

Fig. 4. Response of the yeast ERβ cytosensor to different substances after a 4 h exposure period. Exposure to 17β-estradiol and other substances was started by adding to 200 μl of a yeast culture of the ERβ cytosensor, an aliquot of 1 μl of a stock solution, using either ethanol or DMSO as a solvent as indicated in Tables 1 and 2. Fluorescence was determined after 4 h as described in Section 2 (see Section 2.7). (A) shows dose–response curves of 17β-estradiol (E2), 17α-estradiol (E2α), 4-hydroxyestradiol (4OH-E2), 4-hydroxyestrone (4OH-E1) and estriol; and (B) shows dose–response curves of 17β-estradiol (E2), genistein, genistin, daidzein, daidzin, naringenin (Nar) and 8-prenylnaringenin (8-prenylN). Fluorescence signals are the mean of a triplicate with S.D.

spectively. DES on the other hand showed a two-fold higher potency with the ERβ than with the ERα (see Fig. 5).

Table 2 shows the EC_{50} values and REPs for the phytoestrogens and resorcyclic acid lactones. Based upon these data and the dose–response curves, the ranking of the relative estrogenic potencies of the phytoestrogens and resorcyclic acids with the ERα is as follows: 17β-estradiol ≫ 8-prenylnaringenin > coumestrol > zearalenone ≫ genistein ≫ genistin > naringenin. For ERβ, the ranking is as follows: 17β-estradiol ≫ coumestrol > genistein > zearalenone > 8-prenylnaringenin ≫ daidzein > naringenin > genistin ≫ daidzin. For the phytoestrogens REP values with the ERβ were in general higher than with the ERα, with the exception of 8-prenylnaringenin (see Fig. 5).

Nearly all compounds were tested twice and some of them even three times. The differences in the determined EC_{50} and REP values were very small and negligible (data not shown). The variation in the determined EC_{50} values for the 17β-estradiol reference (see Tables 1 and 2) is mainly due to the solvent used, either ethanol or DMSO [30]. The variation due to inter-experimental differences is less important. To correct for inter-experimental differences and the influence

Table 1
EC_{50} concentration and relative estrogenic potency (REP) of compounds with ERα and ERβ

Compound	EC_{50}[b] (nM) ERα	REP[c] ERα	EC_{50}[b] (nM) ERβ	REP[c] ERβ
17β-Estradiol	0.5–1.0[d]	1.0	0.06–0.25[d]	1.0
17α-Ethynylestradiol	0.5	1.2	0.12	1.0
Diethylstilbestrol	0.6	1.0	0.06	2.0
β-Estradiol 3-benzoate	70	8.6E−3	30	8.3E−3
Hexestrol[a]	2.8	0.36	1.1	9.1E−2
Dienestrol[a]	1.8	0.56	1.1	9.1E−2
Mestranol[a]	9.1	0.11	1.0E2	1.0E−4
Medroxyprogesterone 17-acetate[a]	n.r.	n.r.	n.r.	n.r.
Progesterone[a]	n.r.	n.r.	n.r.	n.r.
Testosterone[a]	>3E4[e]	<3E−5[e]	n.r.	n.r.
19-Nortestosterone[a]	3.0E3	2.8E−4	>5E4	<1.7E−6
Estrone	3	0.2	1.1	0.1
Estriol	1.2E2	5.0E−3	12	5.0E−3
17α-Estradiol	7	9.3E−2	2.8	2.1E−2
2-Hydroxyestradiol	60	1.1E−2	8.5	7.1E−3
4-Hydroxyestradiol	2.5E2	2.6E−3	20	3.0E−3
2-Hydroxyestrone	2.5E2	2.6E−3	2.3 E2	2.6E−4
4-Hydroxyestrone	30	2.2E−2	12.5	4.8E−3
β-Estradiol 3-sulfate[a]	3.4E3	2.6E−4	4.0E2	2.3E−4
β-Estradiol 3-β-D-glucuronide[a]	3.8E3	2.4E−4	1.8E2	5.0E−4
β-Estradiol 17-β-D-glucuronide[a]	>4.0E4	<2.0E−5	>8.0E2	<1.1E−4
Estrone 3-sulfate[a]	1.8E4	5.0E−5	8.0E3	1.1E−5
Estrone β-D-glucuronide[a]	>8E4	<1E−5	>2.8E4	<4E−6

n.r. = no response.

[a] For these compounds DMSO is used as solvent (all the other compounds are tested in EtOH as solvent).

[b] The EC_{50} is the concentration giving a half-maximum response. Exposure was 24 and 4 h for the ERα and ERβ cytosensor, respectively.

[c] The relative estrogenic potency is defined as the ratio between the EC_{50} of E2β and the EC_{50} of the compound.

[d] The range in EC_{50} values of 17β-estradiol. E2β is tested in DMSO and in EtOH as solvent and the EC_{50} for E2β is lower in EtOH as solvent than it is in DMSO [30]. There are also small differences in the EC_{50} values between the different exposures, as all described compounds are not tested in one experiment, but in different exposures during a period of about six months. Here the range of the obtained EC_{50} values is given.

[e] The greater than (>) sign means that this compound does not fully reach a maximum response and therefore the EC_{50} value cannot be determined accurately. The real EC_{50} will be greater than the value that is given in the table. As a result REPs are indicated with the smaller than sign (<).

of the solvent used, each new experiment has its own 17β-estradiol reference and compounds dissolved in ethanol or DMSO are compared with a estradiol reference dissolved in the same solvent.

4. Discussion

The present paper demonstrates the successful construction of yeast cells stably expressing the human estrogen receptors α and β, and producing green fluorescent protein in response to compounds with known estrogenic properties. In addition to the biomolecular controls, this is clearly shown by the very reproducible dose-related transcription activation of the yEGFP gene in both yeast ERα and ERβ cytosensors after exposure to 17β-estradiol (Figs. 1–4). The poor dose–response curves obtained with the ERβ and ERα/ERβ cytosensors after 24 h exposure indicates that the ERβ receptor protein is not as stable as the ERα or that a complex with the ERβ receptor is involved in a quicker signalling pathway.

One of the potential applications of the cytosensors is their use for detecting increased hormonal activity in samples of illegally treated animals. Therefore, it is essential to obtain data on the estrogenic potency of known and putative metabolites and to show the specificity of the assay for estrogenic compounds. The data in Table 1 show that the cytosensors are specific for estrogens, since the gestagens progesterone and medroxyprogesterone 17-acetate, and the androgen testosterone did not show a clear response. Only 19-nortestosterone gave a full dose–response curve, but this compound appears to have both androgenic and estrogenic properties, possibly explaining its excellent anabolic properties requiring both types of hormonal activity. Here, we show for the first time that 19-nortestosterone is a full agonist with ERα and that this compound is relatively more potent with ERα than it is with ERβ (Table 1, Fig. 5). A number of metabolites of 17β-estradiol and estrone showed a clear response, but in general at least a factor 5 to 10^5 less then their parent compounds. There are only small differences between the relative potencies with ERα and ERβ of the natural hormones and their conjugated forms and metabolites. However, like estrone, the conjugated forms E1-3-sulfate and E1-β-D-glucuronide and metabolites 2-OH-E1 and 4-OH-E1 appear to be slightly more potent with ERα than with ERβ. The glucuronidated forms of 17β-estradiol, E2β-3-

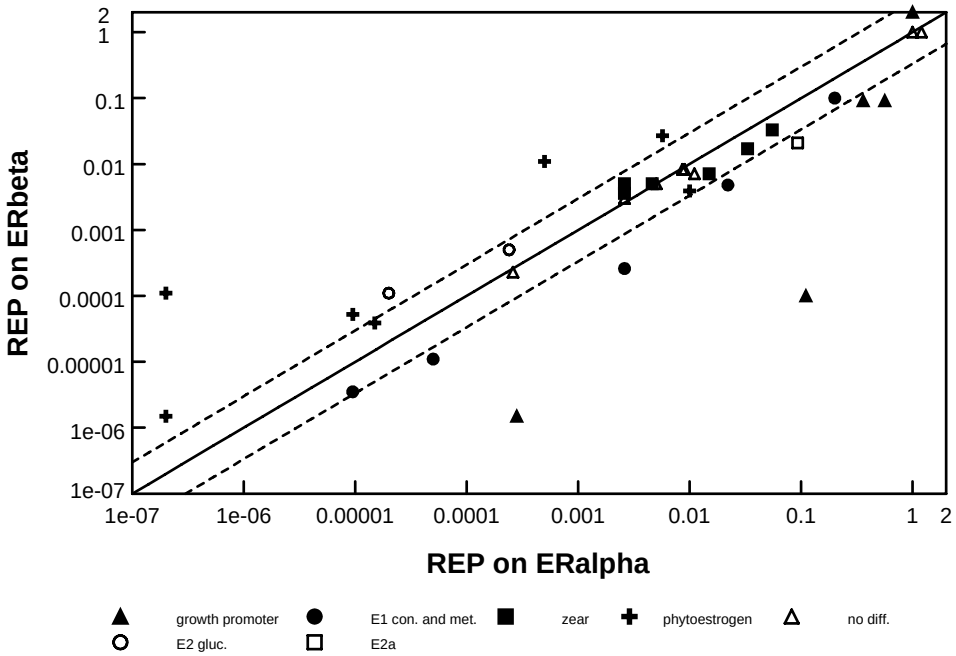

Fig. 5. Relative Estrogenic Potencies (REP) of all compounds with the ERα and ERβ cytosensor. Exposure to 17β-estradiol and other substances was started by adding to 200 μl of a yeast culture of the corresponding cytosensors, an aliquot of 1 μl of a stock solution, using either ethanol or DMSO as a solvent as indicated in Tables 1 and 2. Fluorescence was determined after 4 or 24 h as described in Section 2 (see Section 2.7) and as indicated in Tables 1 and 2. The growth promoters are: DES, hexestrol, dienestrol, mestranol and 19-norT; E1 con. and met. are: E1, 2OH-E1, 4OH-E1, E-3-sulfate and E1-β-D-glucuronide; zear are: z-lenone, z-lanone, α-z-lenol, α-z-lanol, β-z-lenol and β-z-lanol; phytoestrogen are: coumestrol, genistein, genistin, daidzein, daidzin, nar and 8-prenylN; no diff. are: E2β, EE2, E2-benz, E3, 2OH-E2, 4OH-E2, E2-sul and NP; and the E2-gluc. are: E2-3-gluc and E2-17-gluc. The line $x = y$ and the dotted lines $x = 3y$ and $3x = y$ are also shown. Compounds between the lines have more or less the same activity with ERα and ERβ, whereas compounds above the upper dotted line are three times or more active with ERβ than with ERα. Compounds below the lower dotted line are three times or more active with ERα than with ERβ.

β-D-glucuronide and E2β-17-β-D-glucuronide, on the other hand seem slightly more potent with ERβ (see Fig. 5) and 17α-estradiol is more potent with ERα. The present data also show that for testing of, e.g. urine of calves a deconjugation step is a requirement for gaining sensitivity. As 1% deconjugation of the 17β-estradiol conjugates would already result in a REP of 0.01 and as the REPs of these conjugates are much lower than 0.01 and because dose–response curves after a relatively short exposure period of 4 h were similar to those after 24 h, it is most likely that yeast is not able to deconjugate these compounds. For the same reasons the estrogenic activity of β-estradiol 3-benzoate, REP of 8.6E−3, is probably not due to metabolic conversion of the ester. In the present study, no attempts were made to investigate the metabolism of the test compounds by the cells, which may result in activation or deactivation of compounds and may explain possible differences in estrogenic potencies with other test systems [8,9,21–29].

A specific difference in the ligand-binding properties of the two receptors is the affinity for phytoestrogens [8,9]. As described previously, the isoflavones genistein, genistin, daidzein and daidzin, the coumestran coumestrol and the flavonoid naringenin were relatively more potent with ERβ than with ERα (see Table 2 and Fig. 5). Coumestrol and genistein were by far the most potent of these compounds with ERβ. However, 8-prenylnaringenin, a phytoestrogen present in hops, was relatively more potent with ERα than with ERβ and was actually the most potent phytoestrogen with ERα. Until now, comparison of 8-prenylnaringenin with ERα and ERβ was only performed in a receptor binding assay [34], revealing comparable binding activity to both receptor forms. Here we describe for the first time, that 8-prenylnaringen is more potent with ERα than with ERβ, using a yeast transcription activation assay. Although, the data obtained from transcription activation assays are in general comparable with results from radioligand competition binding assays, Kuiper et al. also observed that although coumestrol bound to the ERβ with the same affinity as 17β-estradiol, transcription activation started at 1000 times higher concentrations for coumestrol [8]. Furthermore, ligand-binding assays do not disclose the biological activity of a compound, i.e. whether it is an agonist or an antagonist and therefore, transcription activation assays are supposed to correspond better with effects found in vivo. The characteristics of 8-prenylnaringenin, being a very potent compound that is relatively more potent with ERα than with ERβ, are in agreement with effects described upon the intake of the female flowers of the hop plant, as they have long been used as a preservative. More recently, they have also been included in some herbal preparations for women for

Table 2
EC_{50} concentration and relative estrogenic potency (REP) of resorcyclic acid lactones, phytoestrogens, natural compounds and nonylphenol with ERα and ERβ

Compound	EC_{50}[b] (nM) ERα	REP[c] ERα	EC_{50}[b] (nM) ERβ	REP[c] ERβ
17β-Estradiol	0.5–1.0[d]	1.0	0.06–0.25[d]	1.0
Zearalenone	1.3E2	4.6E−3	20	5.0E−3
Zearalanone	40	1.5E−2	14	7.1E−3
α-Zearalenol	11	5.5E−2	3	3.3E−2
α-Zearalanol	18	3.3E−2	6	1.7E−2
β-Zearalenol	2.3E2	2.6E−3	28	3.6E−3
β-Zearalanol	2.3E2	2.6E−3	20	5.0E−3
Coumestrol[a]	1.4E2	5.7E−3	3	2.7E−2
Genistein[a]	2.0E3	5.0E−4	8	1.1E−2
Genistin[a]	>4E4[g]	<2E−5[g]	2.3E3	3.9E−5
Daidzein[a]	n.r.	n.r.	8.0E2	1.1E−4
Daidzin[a]	n.r.	n.r.	6.0E4	1.5E−6
Enterolactone[a]	n.r.	n.r.	n.r.	n.r.
Enterodiol[a]	n.r.	n.r.	n.r.	n.r.
β-Ecdysone[a]	n.r.	n.r.	n.r.	n.r.
Naringenin[a]	>7E4	<1E−5	2.1E3	5.2E−5
8-Prenylnaringenin[a]	1.0E2	1.0E−2	33	3.9E−3
4-*n*-Nonylphenol[a,e]	n.r.	n.r.	n.r.	n.r.
4-Nonylphenol[a,f]	1.0E2	9.0E−3	30	8.3E−3

n.r. = no response.

[a] For these compounds DMSO is used as solvent (all the other compounds are tested in EtOH as solvent).

[b] The EC_{50} is the concentration giving a half-maximum response. Exposure was 24 and 4 h for the ERα and ERβ cytosensor, respectively.

[c] The relative estrogenic potency is defined as the ratio between the EC_{50} of E2β and the EC_{50} of the compound.

[d] The range in EC_{50} values of 17β-estradiol. E2β is tested in DMSO and in EtOH as solvent and the EC_{50} for E2β is lower in EtOH as solvent than it is in DMSO [30]. There are also small differences in the EC_{50} values between the different exposures, as all described compounds are not tested in one experiment, but in different exposures during a period of about six months. Here the range of the obtained EC_{50} values is given.

[e] Pure 4-*n*-nonylphenol obtained from Riedel-de-Haën.

[f] Technical mixture of *p*-isomers of 4-nonylphenol obtained from Fluka.

[g] The greater than (>) sign means that this compound does not fully reach a maximum response and therefore the EC_{50} value cannot be determined accurately. The real EC_{50} will be greater than the value that is given in the table. As a result REPs are indicated with the smaller than sign (<).

breast enhancement [34,35]. Both applications indicate that 8-prenylnaringenin is an active agonist with ERα. Naringenin, a flavonoid present in citrus fruits, is relatively more potent with ERβ, but compared to 8-prenylnaringenin, this compound is only a weak estrogen. The lignans enterolactone and enterodiol did not show any response with both receptor types and are therefore characterised as being non-estrogenic in our yeast cytosensors.

There are no great differences between the relative potencies of zearalenone and its derivatives with both receptor types (Table 2, Fig. 5). However, compared to the natural phytoestrogens, zearalenone is about as potent as the most potent phytoestrogens coumestrol, genistein and 8-prenylnaringenin, whereas the derivatives α-zearalenol and α-zearalanol are even more potent. The latter is not unexpected, since α-zearalanol is used as a legal growth promoter for cattle breeding in the US [36]. Very similar REPs for this series of resorcyclic acid lactones have been reported by Le Guevel and Pakdel [37], also using a yeast assay with hERα.

Recently, ICCVAM [38] included the environmental pollutant 4-*n*-nonylphenol [CASRN 104-40-5] as a positive control in a set of reference compounds for transcriptional activation assays. In our hands this compound did not show a response in neither of the yeast cytosensors. Others however, have reported 4-*n*-nonylphenol to be active in these type of assays [39,40]. Thorough review of these studies showed that a technical mixture like the one available from Fluka (approximately 85–92.7% of branched isomers) or *p*-nonylphenol (CAS No. 84852-15-3) was used instead of the unbranched nonyl chain. When the 4-nonylphenol technical mixture of Fluka was used, it also showed a dose–response curve in our test with an EC_{50} of 100 nM (see Table 1). Chemical analysis with GC/MS showed that there was actually no 4-*n*-nonylphenol, the aliphatic straight chain, in this technical mixture of Fluka (data not shown). From this, it can be concluded that 4-*n*-nonylphenol is not estrogenic and that the estrogenicity of the technical mixture is due to one or more isomers with a branched side-chain. A similar conclusion was presented by Pedersen et al. studying the induction of plasma vitellogenin in rainbow trout by linear and technical nonyl- and octylphenol [41].

The relatively very high estrogenic potencies of estrone and 4-hydroxyestradiol, as observed in the ER-CALUX assay with T47D cells, giving rise to REPs of 1.0 and 0.45, respectively [42], were not observed with our ERα cytosensor, in which REPs of 0.2 and 0.0026, respectively were observed. This possibly points to important differences between yeast cells and mammalian cells, in for example

the metabolism and absorption of compounds and the transcription activation pathway. However, as shown in the case of biochanin A and estrone for metabolism [8,42] and for transcription activation [22,23], similar differences exist between different mammalian cell lines. This yeast assay therefore represents another appropriate assessment of the relative activity of various estrogens.

In general the response obtained for E2β with the ERα receptor is in a similar dose-range as described for yeast by other authors [9,27,37,40]. The lower maximum response for ERβ, as compared to ERα, has been reported for mammalian cells as well [22,23], but the observation that the yeast cells expressing ERβ show a response at lower concentrations of 17β-estradiol has not been observed previously. Morito et al. [9] used a yeast estrogen assay and found that the dose–response curve obtained with 17β-estradiol for the yeast expressing the ERα was similar to the one obtained with the yeast expressing the ERβ. In Chinese hamster ovary cells (CHO) transiently transfected with an ERα or ERβ expression construct and an ERE-based reporter construct, the transcriptional activity after exposure to 17β-estradiol of ERβ was only about 50% of the activity observed with ERα. However, in this study with CHO cells, half-maximal activation was reached at lower concentrations for ERα than for ERβ, giving EC_{50} values for 17β-estradiol of 0.04 and 0.3 nM, respectively [7,22]. At the same time, this latter study also revealed that the ability of ERβ to function as an estrogen-dependent transcriptional activator is highly dependent on the cellular context. It was shown that in human endometrial cancer (HEC-1) cells and MDA-231 breast cancer cells transcription activation after exposure to 17β-estradiol by ERβ was respectively only about 15% and less than 5% of the transcriptional activity by ERα. In this case, half-maximal activation was again reached at lower concentrations for ERα than for ERβ. Similar results were obtained with human embryonal kidney 293 cells.

Another interesting observation is the apparent lower response in the cells expressing both receptor types (Fig. 3), indicating that either ERβ is dominating in the possible heterodimers or that ERβ prevents the formation or activity of the ERα homodimer. Thus, just like ER(beta)cx [17], it seems that ERβ is a dominant repressor of ERα function, at least in the case of yeast. These differences between both receptor types might reflect characteristics of the ERβ and support the hypothesis that ERβ may have a role in protection against hyperproliferation and carcinogenesis. Therefore, the repressor (ERβ) has to be more sensitive to 17β-estradiol, explaining the lower EC_{50} for ERβ than for ERα, but the maximum response should be lower, repressing the activity of the ERα. Even a lower stability of the repressor (ERβ) could support this hypothesis, as repressor activity is not needed all the time in a cell. Also, the inability to obtain a full-length cDNA of the ERβ without any mutations (see Section 2.4) from mRNA isolated from the human T47D breast cancer and Caco-2 colon cancer cells, might point at a contribution of an inactivated ERβ function, to the origin of the cancer. At the same time, this observation stresses the need to reinvestigate the identity and functionality of the estrogen receptors in the different bioassays used for testing estrogenic activity.

In conclusion, both the ERα cytosensor and ERβ cytosensor show clear dose–response curves when exposed to estrogenic compounds. These yEGFP assays are not only very sensitive, as shown by EC_{50} values for E2β of 0.5 and 0.06 nm for the ERα and ERβ cytosensor, respectively, but are also very rapid, convenient, reproducible and most likely more robust than cell-lines. Both cytosensors can be used to study the estrogenicity of different compounds in order to determine the relative estrogenic potency of these compounds. Since good dose–response curves can be obtained after only 4 h of exposure, the often questioned permeability of the yeast cell wall does not seem to be an obstacle in our yeast estrogen assay.

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