

ER β : identification and characterization of a novel human estrogen receptor

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Abstract A novel estrogen receptor (hereinafter referred to as ER β) was cloned using degenerate PCR primers. A comparison of the amino acid sequence of ER β with the 'classical' ER (ER α) shows a high degree of conservation of the DNA-binding domain (96%), and of the ligand-binding domain (58%). In contrast, the A/B domain, the hinge region and the F-domain are not conserved. Northern blot analysis revealed that ER β is expressed in human thymus, spleen, ovary and testis. Transient transfections of an ER β expression construct together with an ERE-based reporter construct in CHO cells clearly demonstrated transactivation of ER β by 17 β -estradiol. In addition, the ER α antagonist ICI-164384 is a potent antagonist for ER β as well. Interestingly, the level of transactivation by 17 β -estradiol is higher for ER α than for ER β , which may reflect suboptimal conditions for ER β at the level of the ligand, responsive element or cellular context.

Key words: Estrogen receptor; 17 β -Estradiol; Anti-estrogens; Transactivation; Human

1. Introduction

The estrogen receptor (ER α) is a member of the superfamily of nuclear receptors which are able to transduce extracellular signals (small lipophilic molecules) into transcriptional responses [1]. In general, nuclear receptors have a modular structure with six distinct regions, A–F. Of these, region C (corresponding to the DNA-binding domain) and region E (corresponding to the ligand-binding domain) are evolutionarily conserved [2]. Steroid hormone receptors are distinct from other nuclear receptors in a number of respects including the nature of their ligands, their association (in the unliganded state) with a repertoire of heat-shock proteins, and the fact that they bind to hormone response elements as homodimers [3]. Since the cloning of the ER α cDNA [4,5], a great deal of detailed knowledge regarding its mechanism of action has been obtained (see e.g. [6–8]). Basically, ER α dimerizes upon ligand binding and the homodimer then binds to estrogen response elements (EREs) in the transcriptional control regions of target genes. In addition, a number of pathways are now known to influence ER functioning: e.g. different conformations induced by different ligands, modifications of the receptor by phosphorylation and interactions of the receptor with other transcription factors [8–10]. In recent years, ER α has become an important target for the identification of com-

pounds which may be effective in estrogen-dependent therapies such as hormone-replacement therapy and cancer.

Here, we report the identification and characterization of a novel human estrogen receptor (designated ER β), which is highly homologous with the 'classical' ER α and has an overlapping but non-identical tissue distribution. We demonstrate that this novel receptor is functional in that it interacts with (anti-)estrogens and is able to modulate estrogen-responsive reporter gene expression.

2. Materials and methods

2.1. RT-PCR, RACE-PCR and primers

One microgram of total RNA (from EBV-stimulated human peripheral blood leukocytes) was reverse transcribed in a 20 μ l reaction containing 50 mM KCl, 10 mM Tris-HCl pH 8.3, 4 mM MgCl₂, 1 mM dNTPs (Pharmacia), 100 pmol random hexanucleotides (Pharmacia), 30 units RNase inhibitor (Pharmacia) and 200 units M-MLV reverse transcriptase (Gibco BRL) at 37°C for 30 min followed by 5 min at 100°C. Two degenerate oligonucleotides were based on conserved regions of the DNA-binding domains (primer 1) and the ligand-binding domains (primer 2) of the human steroid hormone receptors. PCR reactions (100 μ l) contained 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatine (w/v), 3% DMSO, 1 μ g of each primer and 2.5 units of Amplitaq DNA polymerase (Perkin Elmer). The PCR protocol was: 4 min at 94°C, then 36 cycles (30 s 94°C, 30 s 45°C, 1 min 72°C) and finally 7 min at 72°C. All other PCR reactions were performed with an annealing temperature of 55°C and with 200 ng of each primer. Fragments of interest were cut out of a 1.5% agarose gel, reamplified and cloned using the TA-cloning kit (Invitrogen) and sequenced using a T7 DNA sequencing kit (Pharmacia).

RACE (rapid amplification of cDNA ends) PCR reactions were performed using a Marathon-ready kit (Clontech) with testis cDNA as template.

- Primer 1: 5'-GGIGA(C/T)GA(A/G)GC(A/T)TCIGGITG(C/T)C-A(C/T)TA(C/T)GG-3'
 Primer 2: 5'-AAGCCTGG(C/G)A(C/T)IC(G/T)(C/T)TTIGCC-CAI(C/T)TIAT-3'
 Primer 3: 5'-TGTTACGAAGTGGGAATGGTGA-3'
 Primer 4: 5'-GGC(C/G)TCCAGCATCTCCAG(C/G)A(A/G)CA-G-3'
 Primer 5: 5'-TCTTGTCTGGACAGGGATG-3'
 Primer 6: 5'-GGAAGCTGGCTCACTTGCTG-3'

2.2. Northern blot analysis

Human multiple tissue Northern blots (Clontech) were prehybridized for at least 30 min at 65°C in 0.5 M phosphate buffer pH 7.5 with 7% SDS. A ER β -specific PCR fragment was generated using primer 2 in combination with primer 3. This probe was ³²P-labelled using a Decaprime kit (Ambion), denatured by boiling, added to the prehybridization solution and incubated overnight at 65°C. Washing conditions were: 3 \times SSC at room temperature, followed by 3 \times SSC at 65°C and then 1 \times SSC at 65°C.

2.3. Cloning of ER β

Approximately 400 000 recombinant phages of a human testis

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The nucleotide sequence of the cloned human ER β has been assigned the accession number X99101.

cDNA library in λ gt11 (Clontech HL1010b) were screened. Replica filters (Hybond-N, Amersham) were made as described by the manufacturer and hybridized with the ER β -specific probe (see Section 2.2). Filters were prehybridized in a solution containing 0.5 M phosphate buffer (pH 7.5) and 7% SDS at 65°C for at least 30 min. Probe-labelling and hybridization were as described in Section 2.2. Filters were washed in 0.5 \times SSC/0.1% SDS at 65°C. Positive clones were plaque-purified and fragments generated by PCR were cloned and sequenced. For verification of the nucleotide sequences the complete cDNA insert was generated using the proof-reading *Pfu* polymerase (Stratagene), cloned and both strands were sequenced.

In order to clone sequences downstream of exon 7, primer 4, based on the ER α AF-2 region was used in combination with ER β -specific primer 5 (nucleotides 936–955 in Fig. 1A) and then nested with primer 6 (nucleotides 1130–1149 in Fig. 1A), using testis cDNA (Marathon-ready, Clontech) as template. A specific fragment was analyzed as described above. The remainder of the carboxy-terminus was cloned using RACE PCR reactions on the same testis cDNA. First primer 5 was used together with the AP1 primer provided in the kit, then a nested PCR was performed using primer 6 together with the CDS primer from the kit. Fragments that were obtained were cloned and sequenced.

2.4. Cell culture, transient transfections and transactivation assay

Chinese hamster ovary cells (CHO K1; CCL61) were cultured in phenol red-free M505 medium (a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM, Gibco) and nutrient medium F12 (Ham's F12, Gibco)) supplemented with 5% charcoal-treated bovine calf serum (Hyclone) and antibiotics. The ER β reading frame was cloned in the mammalian expression vector pNGV1, a modification of pKCR [11] containing the SV40 early promoter and the IgG and MuLV enhancers. The reporter expression vector was based on the rat oxytocin gene regulatory region position –363/+16 [12] linked to the firefly luciferase gene. CHO cells (1×10^5) were seeded in 6-well Nunclon tissue culture plates and DNA (1 μ g of both receptor and reporter constructs and 250 ng β -galactosidase vector in 250 μ l Optimem, Gibco BRL) was mixed with an equal volume of lipofectin reagent (7 μ l in 250 μ l Optimem) and allowed to stand at room temperature for 15 min. This solution was mixed (1:1) with Optimem and 1 ml of this mixture was added to each well after washing the cells (serum-free M505). After a 5-h incubation the cells were washed (phenol red-free M505 with 5% charcoal-treated bovine calf serum) and incubated overnight at 37°C. After 24 h hormone was added to the medium. 17 β -Estradiol (Org2317) was obtained from Organon, Oss, The Netherlands, ICI-164384 was kindly provided by Dr. A.E.

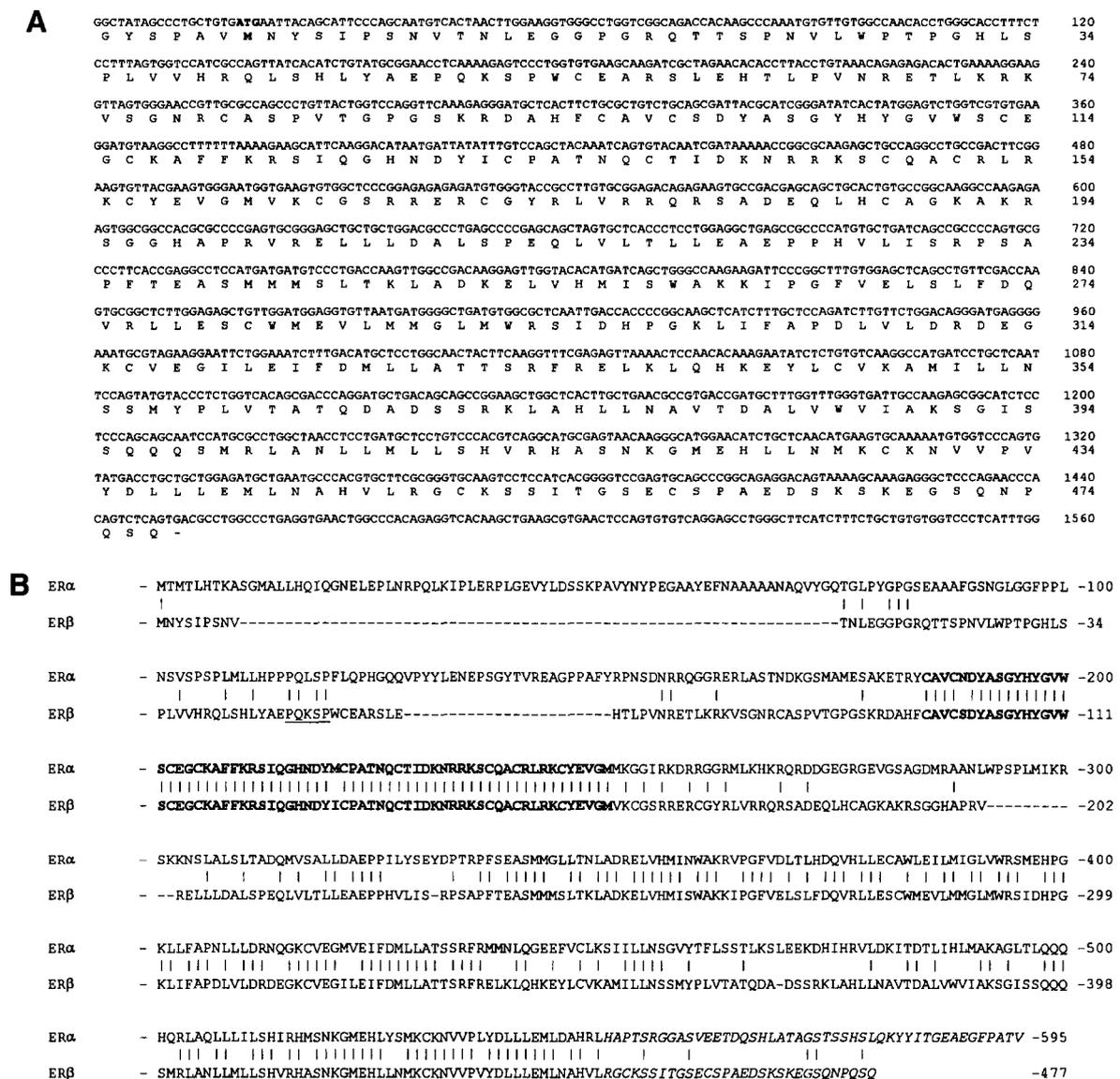


Fig. 1. (A) Nucleotide sequence and deduced amino acid sequence of ER β . (B) Alignment of the amino acid sequences of the human ER β with the human ER α . The DNA-binding domains are shown in bold, the consensus sequence for MAPK-mediated serine phosphorylation is underlined and the F-domains are shown in italics.

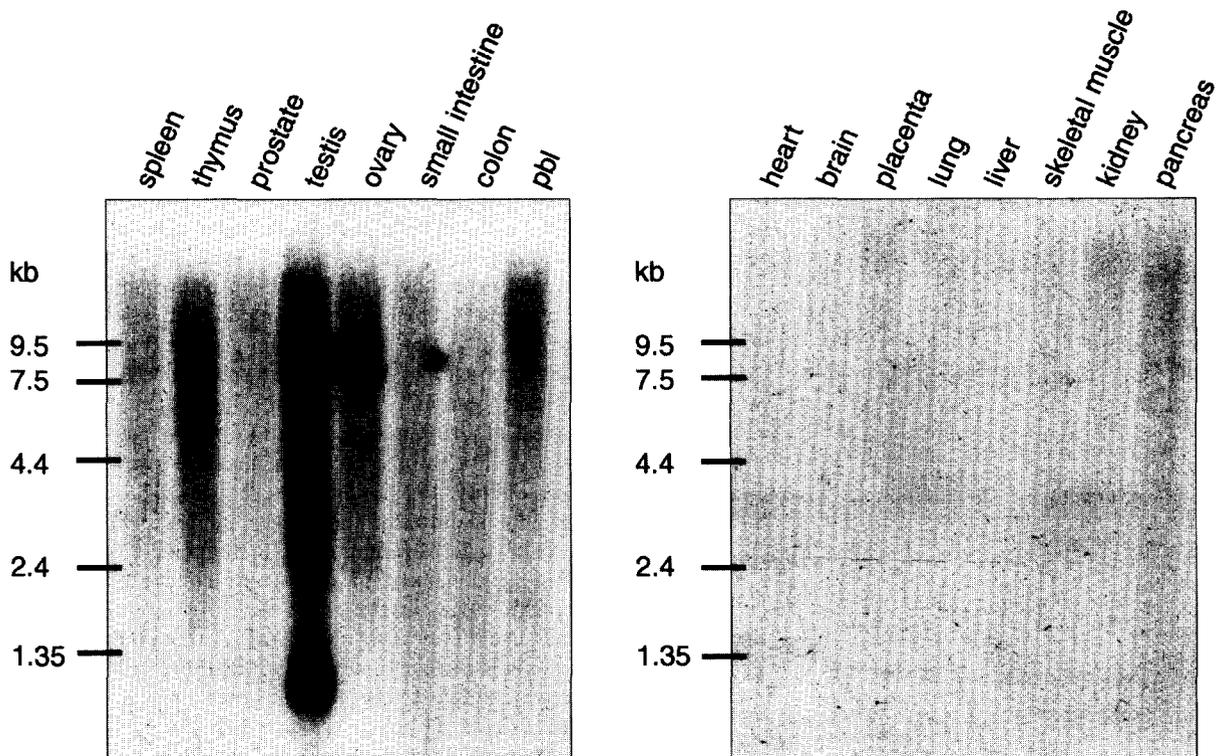


Fig. 2. Tissue distribution of ER β . Each lane contains 2 μ g of poly A⁺ RNA isolated from the human tissues indicated at the top (PBL, peripheral blood leukocytes). RNA size markers are indicated on the left.

Wakeling, Zeneca Pharmaceuticals, Macclesfield, UK. Cell extracts were made 48 h post-transfection by the addition of 200 μ l lysis buffer (0.1 M Na₂HPO₄, 0.2% Triton X-100). 20 μ l sample was added to 50 μ l luciferase assay reagent (Promega). Light emission was measured in a Topcount (Packard). Beta-galactosidase activity was measured using a Galacto-light plus kit (Tropix) to correct for differences in transfection efficiency.

3. Results

Using degenerate primers (see Section 2) we cloned a PCR fragment which corresponds to a novel receptor (designated ER β) which is highly homologous with the classical estrogen receptor (ER α). Part of this fragment (corresponding to nucleotides 484–815 in Fig. 1) was used as a probe to determine the tissue distribution of ER β by Northern blot analysis. Fig. 2 shows that ER β is expressed in thymus, testis and ovary (approx. 8 kb and 10 kb transcripts) and very faintly also in spleen. In addition, a relatively abundant transcript of 1.3 kb is present in testis. Screening of a human testis cDNA library resulted in the identification of two identical positive clones which contained a large part of the ER β reading frame (nucleotides 1–1265 in Fig. 1A). At the 5' end, an in-frame ATG codon (nucleotides 19–21) is present, but since the clone lacks an in-frame stop codon further upstream, we cannot exclude the possibility that upstream ATG translation initiation codons may be present. At the 3' end, the homology with the ER α is lost abruptly at a site which corresponds to the splice site between exon 7 and exon 8 in ER α , suggesting that this cDNA is derived from an incompletely spliced transcript. In order to obtain nucleotide sequences downstream of this splice site, testis cDNA (Marathon-ready, Clontech) was used as a template to generate a RACE PCR fragment that

was cloned and sequenced. It was shown to contain the carboxy-terminus of ER β , including AF-2, an F-domain followed by a stop codon and part of the 3' untranslated region. A comparison of the complete ER β and ER α amino acid sequences is shown in Fig. 1B.

The high degree of conservation of the DNA-binding domain (96%) and the relatively high degree of conservation of the ligand-binding domain (58%), as compared to currently reported estrogen receptor-related receptors ERR1 and ERR2 [13], suggested that ER β could bind estrogenic hormones and activate transcription of ERE-containing promoters. To experimentally test this hypothesis, CHO cells were transfected with an ER β (or ER α) expression construct together with a well-known ER α -responsive reporter plasmid (rat oxytocin promoter-luciferase, RO-luc [14]) and treated with 17 β -estradiol. Fig. 3A clearly shows that ER β can activate transcription of RO-luc in a hormone-dependent manner, although the level of induction is higher for ER α . In addition, ER β is able to activate transcription of a reporter construct containing three synthetic EREs (not shown). Fig. 3A also shows that the ER α antagonist ICI-164384 (ICI) is a potent antagonist for both ER α and ER β . Fig. 3B shows a dose-dependent transactivation of ER α and ER β by 17 β -estradiol, indicating that both receptors can be activated at very low hormone concentrations. However, half-maximal activation is reached at lower concentrations of 17 β -estradiol for ER α than for ER β .

4. Discussion

The current report describes the identification of ER β as a functional estrogen receptor which can interact with the re-

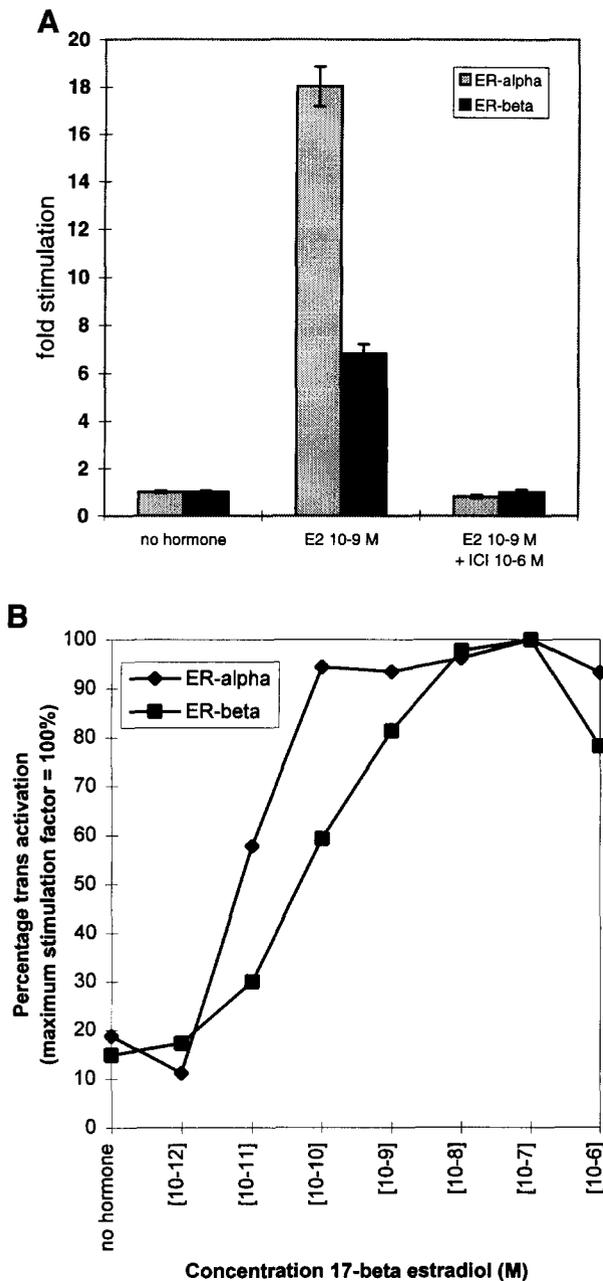


Fig. 3. (A) Effect of 17 β -estradiol (E2) alone or in combination with the anti-estrogen ICI-164384 (ICI) on the transcriptional activity of ER α (gray bars) and ER β (black bars) using an ERE-based reporter gene. Indicated is the fold induction of the luciferase activity of cells receiving no hormone. All luciferase activities were determined in triplicate and were normalized for differences in transfection efficiency by measuring β -galactosidase activity in the same lysate. (B) Concentration-dependent transactivation of ER α and ER β by 17 β -estradiol. Transactivation at 10⁻⁷ M was arbitrarily set at 100%.

ference ER α agonist (17 β -estradiol) as well as a widely used antagonist (ICI-164384). ER β was demonstrated to activate transcription of ERE-containing promoters. Consequently, a number of experimental *in vitro* and *in vivo* data, including those concerning the ER α knock-out mice [15], will have to be re-evaluated.

A comparison of ER β with ER α reveals a number of interesting aspects. The tissue distribution of ER β appears to be

restricted to thymus, spleen, ovary and testis. However, the initial PCR fragment was cloned from peripheral blood lymphocytes, whereas no signal is detected on Northern blot. Therefore, it may be that the sensitivity of Northern blot analysis is insufficient to detect ER β expression in some tissues. It is not likely that the probe and the conditions used allow hybridization with ER α . Hybridization of the same blots with a probe corresponding to part of the 3' untranslated region of ER α revealed a 6.5 kb band in almost all tissues, especially ovary, testis, prostate and skeletal muscle (not shown). The ligand-binding domain is relatively well conserved (58%) and our data demonstrate that as a result, ER β closely resembles ER α with respect to the binding of 17 β -estradiol and ICI-164384. This suggests that there is a high degree of structural conservation of the ligand-binding pocket(s). Although both receptors interact with 17 β -estradiol with high affinity, Fig. 3B suggests that ER α has a higher affinity for this ligand than ER β . The difference with respect to the level of induction may relate to the nature of the ligand, the responsive element or to the cellular context (co-activators/repressors), which may not have been optimal for ER β in the current co-transfection assay. Although the DNA-binding domain is extremely well conserved (96%), ER β and ER α may activate different sets of target genes. Since not only the P-box but also the D-box is conserved, it can be envisaged that in addition to forming homodimers, ER β and ER α may form heterodimers thereby influencing each other's transcriptional activity.

The A/B domain, the hinge (domain D) and the F-domain are poorly conserved. Interestingly however, the ER β A/B domain contains a consensus sequence for MAPK-mediated serine-phosphorylation (PQKSP in ER β , underlined in Fig. 1B) which is also present and functional in the ER α A/B domain [16]. The lack of conservation of the A/B domain suggests important differences between both receptors with respect to their AF-1 activity and with respect to interactions of co-activators and/or repressors with this region. The lack of conservation of the F-domain is also striking in view of its suggested role in modulating the extent of the agonistic activity of compounds like 4-OH-tamoxifen in ER α [17].

In conclusion, the cloning of ER β adds to the complexity of (tissue-specific) estrogenic effects. It can be envisaged that it will be worthwhile to dissect ER β pathways from ER α pathways as well as to identify compounds which specifically interact with ER α or ER β .

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