Identification of novel estrogen receptor α antagonists

Dalei Shao a,∗, Thomas J. Berrodin a, Eric Manas b, Diane Hauze b, Robert Powers c,1, Ashok Bapat a, Daniel Gonder a, Richard C. Winneker a, Donald E. Frail a,2

a Women’s Health and Bone, Wyeth Research, 500 Arcola Road, RN2294, Collegeville, PA 19426, USA
b Chemical and Screening Sciences, Wyeth Research, Collegeville, PA, USA
c Chemical and Screening Sciences, Wyeth Research, Cambridge, MA, USA

Received 24 September 2003; accepted 16 January 2004

Abstract
We have identified novel estrogen receptor alpha (ERα) antagonists using both cell-based and computer-based virtual screening strategies. A mammalian two-hybrid screen was used to select compounds that disrupt the interaction between the ERα ligand binding domain (LBD) and the coactivator SRC-3. A virtual screen was designed to select compounds that fit onto the LxxLL peptide-binding surface of the receptor, based on the X-ray crystal structure of the ERα LBD complexed with a LxxLL peptide. All selected compounds effectively inhibited 17β-estradiol induced coactivator recruitment with potency ranging from nano-molar to micromolar. However, in contrast to classical ER antagonists, these novel inhibitors poorly displace estradiol in the ER-ligand competition assay. Nuclear magnetic resonance (NMR) suggested direct binding of these compounds to the receptors pre-complexed with estradiol and further demonstrated that no estradiol displacement occurred. Partial proteolytic enzyme digestion revealed that, when compared with 17β-estradiol- and 4 hydroxy-tamoxifen (4-OHT) bound receptors, at least one of these compounds might induce a unique receptor conformation. These small molecules may represent new classes of ER antagonists, and may have the potential to provide an alternative for the current anti-estrogen therapy.© 2004 Elsevier Ltd. All rights reserved.

Keywords: Estrogen receptor; Coactivators; SRC; Antagonist

1. Introduction
Estrogen receptors (ERα and ERβ) are members of the nuclear receptor superfamily. Nuclear receptors are ligand-regulated transcription factors. Most nuclear receptors share structural similarity characterized by several functional domains. Like other nuclear receptors, the full length ERs consists of a ligand independent transactivation domain AF1 (activation function 1) at the N-terminus, a central DNA binding domain (DBD), and a C-terminal ligand binding domain (LBD). The LBD also contains a ligand dependent transactivation domain (AF2). Binding of ligand induces an alteration of the LBD conformation, which determines the ability of the LBD to recruit coactivators, a family of proteins that are essential for receptor-mediated transactivation (reviewed in [1]). A number of coactivators have been identified. Some of the most well characterized coactivators belong to the p160 family of coactivators, including the steroid receptor coactivator 1 (SRC-1/NcoA1) [2], SRC-2/GRIP/TIF2 [3,4], and SRC-3/AIB1/RAC3/ACTR/p/CIP [5–9]. Coactivators recognize the agonist-bound nuclear receptor through the nuclear receptor interaction domain (NRID), which contains one or more conserved short signature motifs, LxxLL [10]. The X-ray structure of the ERα LBD co-crystallized with diethylstilbestrol (DES) and a GRIP1 LxxLL peptide reveals a hydrophobic coactivator docking cleft formed by helices 3, 4, 5 and 12 of the receptor upon agonist binding [11]. The integrity of this interaction interface is essential for coactivator binding and subsequent ligand dependent transactivation.

ERs plays important roles in diverse physiological pathways. Estradiol and compounds that modulate ERs activity are currently being used to treat a variety of diseases including menopausal symptoms, such as hot flush, breast cancer [12] and osteoporosis (review in [13]). All of the known ERs ligands bind exclusively to the ligand binding pocket in the LBD, and affect coactivator recruitment. In particular, coactivator SRC-3 was found to be amplified or overexpressed in over 60% of human primary breast cancer...
patients [5,14]. SRC-3 has been shown to interact with ERα endogenously [15], and down regulation of SRC-3 message level decreases estrogen dependent growth of human breast cancer MCF7 cells [16]. These data provide biological evidences corroborating that recruitment of coactivator SRC-3 is an essential event for ERα to function in breast tissue. In an attempt to identify novel ERα inhibitors, we have designed experiments to identify small molecules that block the 17β-estradiol induced interaction between SRC-3 and ERα without displacing the agonist. This type of ERα modulator may provide an alternative for cancer therapy and might not be compromised by the development of hormonal resistance often seen with current antiestrogen therapy for breast cancer (reviewed in [12]).

2. Materials and methods

2.1. High throughput mammalian two hybrid (M2H) assay

A modified mammalian two-hybrid assay was performed using ERα LBD cloned into the GAL4 DBD (DNA binding domain) plasmid pM (Clontech) and full-length SRC-3 cloned into pCAG3.1 (Invitrogen) along with a GAL4 repressor luciferase (GRE-Luc) reporter. The endogenous LBD cloned into the GAL4 DBD (DNA binding domain) plasmid pM (Clontech) and full-length SRC-3 cloned into pCAG3.1 (Invitrogen) along with a GAL4 repressor luciferase (GRE-Luc) reporter. A control assay consisted of the same GRE-Luc transactivation function of SRC-3 was exploited in a minimal number of docking events. Once docked, the pharmacophore points (or a subset of them) were matched to pre-defined DOCK site points in the binding interaction of each individual conformer with the target structure, and site points for this binding pocket were determined from MCSS2SPTS [17]. These site points were then augmented with site points from the NR-box peptide. Consistent with the topology of the “charge-clamp” binding pocket, the site points at the bottom of the groove were labeled as hydrophobes, whereas those on either end were labeled as donors and acceptors, respectively.

Virtual screening of the available chemicals directory (ACD) database (MDL Information Systems Inc., 1997) was performed using the PharmDOCK method [17] as implemented in the DOCK4.0.1 program [18]. Briefly, ligand flexibility was included by docking ensembles of pre-computed conformers from a conformationally expanded database. The ensembles were pharmacophore-based in that conformers of the same or different molecules were overlaid by their largest three-dimensional pharmacophore. During the docking, the pharmacophore points (or a subset of them) were matched to pre-defined DOCK site points in the binding region of the target structure to orient the ensemble. This allowed for a large sampling of conformer space with a minimal number of docking events. Once docked, the interaction of each individual conformer with the target molecule was scored. Chemically-labeled DOCK site points were generated in an automated fashion using the script MCSS2SPTS [19].

2.4. Estrogen receptor competition assay

Human ERα ligand binding domain (domains DEF) was overexpressed in E. coli strain BL21 (DE3). Transformed bacteria were maintained in LB medium containing 100 mg/ml ampicillin. A 100 ml overnight culture was inoculated into 500 ml of medium and grown to OD600 =0.6. One millimole IPTG was added and the culture was further incubated for an additional 3 h. Cells were subsequently harvested and resuspended in 100 ml buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, and 30% glycerol). The cell suspension was then sonicated for 1 h at 25°C. Plates were washed three times for five minutes each with binding buffer (50 mM TRIS pH8, 150 mM NaCl, 1 mM DTT, 0.01% NP40, 0.1% bovine serum albumin). FLAG-ERα protein was added plus the treatments and incubated 15 h at 4°C. Plates were washed as above and anti-FLAG antibody conjugated to horseradish peroxidase was added for 1 h at 25°C. After an additional wash, Supersignal ELISA substrate (Pierce) was added and chemiluminescence was measured on a Victor plate reader (Perkin Elmer).

2.3. Virtual screen

The X-ray crystal structure of the ERα ligand binding domain complexed with diethylstilbestrol (DES) and a NR-box II peptide [11] was used to perform the virtual screen. The NR-box peptide was removed from the structure, and site points for this binding pocket were determined from MCSS2SPTS [17]. These site points were then augmented with site points from the NR-box peptide. Consistent with the topology of the “charge-clamp” binding pocket, the site points at the bottom of the groove were labeled as hydrophobes, whereas those on either end were labeled as donors and acceptors, respectively.

Virtual screening of the available chemicals directory (ACD) database (MDL Information Systems Inc., 1997) was performed using the PharmDOCK method [17] as implemented in the DOCK4.0.1 program [18]. Briefly, ligand flexibility was included by docking ensembles of pre-computed conformers from a conformationally expanded database. The ensembles were pharmacophore-based in that conformers of the same or different molecules were overlaid by their largest three-dimensional pharmacophore. During the docking, the pharmacophore points (or a subset of them) were matched to pre-defined DOCK site points in the binding region of the target structure to orient the ensemble. This allowed for a large sampling of conformer space with a minimal number of docking events. Once docked, the interaction of each individual conformer with the target molecule was scored. Chemically-labeled DOCK site points were generated in an automated fashion using the script MCSS2SPTS [19].

2.2. SRC/ERα interaction assay (SEIA)

FLAG-tagged ERα was produced in a baculovirus infected sf9 cell expression system. The nuclear receptor interaction domains (NRIDs) of SRC-1 (amino acids 613–773), SRC-2 (amino acids 618–766), and SRC-3 (amino acids 601–762) were expressed as GST fusion proteins in the E. coli strain BL21 DE3. GST-NRID protein was bound to anti-GST antibody coated 96 well plates (Pierce) for 1 h at 25°C. Plates were washed three times for five minutes each with binding buffer (50 mM TRIS pH8, 150 mM NaCl, 1 mM DTT, 0.01% NP40, 0.1% bovine serum albumin). FLAG-ERα protein was added plus the treatments and incubated 15 h at 4°C. Plates were washed as above and anti-FLAG antibody conjugated to horseradish peroxidase was added for 1 h at 25°C. After an additional wash, Supersignal ELISA substrate (Pierce) was added and chemiluminescence was measured on a Victor plate reader (Perkin Elmer).
20's twice, and insoluble material was pelleted by centrifugation at 13,000 rpm for 20 min. The supernatant was aliquoted and stored at −80 °C. The optimal amount of ERαs containing lysate for the binding assay was determined for each preparation, and dilution was made accordingly for each preparation, and dilution was made accordingly in the assay buffer (1 mM EDTA in 1X DPBS (without Ca²⁺ and Mg²⁺)). For each reaction, 100 µL of ERα extract was added to each well of a high binding masked microtiter plate (Wallac), with 10 µL of 10⁻⁶ estradiol (2 mM final concentration) and 10 µL of unlabeled test compound. The reaction was incubated at RT for 6–18 h. The plate was then washed three times with assay buffer. For measuring the radioactivity, 135 µL of scintillant (Optiphase supermix) was added to each well, and the plate was gently agitated for a few minutes before counting. Data was collected on Beckman LS6500 and analyzed using GraphPad Prism one site competition method.

2.5. Nuclear magnetic resonance (NMR)

A 40 mM stock solution of each compound in 100% DMSO-d₆ was used to prepare either a 50 µM or 100 µM nuclear magnetic resonance (NMR) sample of the free compound in 50 mM Tris buffer, pH 7.0, containing 150 mM NaCl, 1 mM DTT with a final DMSO concentration of 1%. A 260 µM stock solution of ERα, in a 1:1 molar complex with a soluble in house estrogen (17β-estradiol was not soluble in this solution), in the above buffer, was used to titrate each NMR sample. The ERα titration points consisted of 2, 4, 10, 15, 25, and 50 µM or 4, 8, 20, 30, 50, and 100 µM additions of ERαs to each compound.

All NMR spectra were recorded at 25 °C on a Bruker 600 MHz AVANCE spectrometer equipped with a triple-resonance, z-axis gradient cryoprobe. The 1D NMR spectra were collected with a sweep-width of 1092.8 Hz with 16K points. A total of 128 scans were collected with a re-cycle delay of 1.8 s. The data was processed and displayed using XWINNMR V3.0 software with a skewed sine-bell apodization function and one zero-filling.

2.6. Partial proteolytic digestion assay

In vitro translated full length receptor (Promega): 35S labeled ERα was produced in rabbit reticulocyte lysate using the TNT coupled transcription/translation system (Promega). The ERαs receptor was incubated with ligands (10nM 17β-estradiol or 4-OHT, or 10 µM cypds) for 1 h at 25 °C. Trypsin (Sigma) was then added at 50 µg/ml and time period indicated. Digestions were terminated with the addition of NuPage (Invitrogen) sample loading buffer and heated to 75 °C for 10 min. Samples were loaded on 4–12% NuPage Bis-Tris gels (Invitrogen) in MES running buffer to facilitate the separation of low molecular-weight fragments. Gels were fixed in 50% methanol/10% acetic acid, enhanced with Amplify fluorography reagent (Amersham), dried and exposed to film overnight.

2.7. Statistical analysis

Data were run using a statistical program SASexcel for one-way ANOVA or non-linear dose responses. Least significant difference (LSD) tests were used to generate the P-values.

3. Results

3.1. Identification of a novel class of ER antagonist through a mammalian two-hybrid screening assay

It has been well established that ERαs LBD interacts with a number of coactivators including SRC-3. This receptor/coactivator interaction is essential for ERαs to fully transactivate its target gene expression. A modified mammalian two-hybrid (M2H) assay was developed using hERαLBD fused to Gal4DBD and the full length SRC-3. In the presence of 17β-estradiol, ERαs LBD will interact with SRC-3, leading to the activation of the reporter gene. Addition of an excess amount of ERα antagonists, such as ICI182,780 abolishes the interaction, hence no activation occurs. Using this method in a high throughput format, we have identified several classes of ERα antagonists. One such antagonist, ERI-5, shows no structural similarity with known anti-estrogens, such as 4-hydroxy-tamoxifen (4-OHT) or ICI182,780 (Fig. 1). This small molecule antagonized 17β-estradiol mediated recruitment of SRC-1 or SRC-3 to the ERα receptor with an IC50 of 5.5 µM in the M2H assays in COS7 cells (Fig. 2a). ERI-5 also inhibited estrogen activity in the M2H using SRC-3-VP16 fusion instead of SRC-3 alone, and showed no inhibitory effect on the Gal4DBD-SRC-3 one hybrid assay (data not shown). These data suggested that the inhibitory effect of ERI-5 was due to the inhibition of ERαs but not SRC-3. The antagonist activity of ERI-5 was also confirmed when tested in a non-cell-based SRC/ERα interaction assay (SEIA) using recombinant ERαs LBD and SRC NRID (see Section 2) (Fig. 2b). The compound also well antagonized the ability of ERI-5, but only weakly antagonized that of the progesterone receptor (PR) at 100 µM, to recruit coactivator (Fig. 2c), suggesting it was mostly estrogen receptor selective.

3.2. Identification of a novel class of ERα antagonist through a computer based virtual screen

The co-crystallization of ERαs LBD bound with DES in the presence of LxxLL peptide provided a structural base of the interaction interface between the agonist-bound receptor and the coactivator [11] (Fig. 3a). We have attempted to identify small molecules that mimic the LxxLL peptide in...
Fig. 1. Chemical structures of estrogen receptor antagonists.

contacting the ERs coactivator interaction surface (Fig. 3b). This would permit us to identify novel ERs inhibitors that would compete for the binding of coactivators. We used this model to screen against the Available Chemical Directory database (MDL Information Systems Inc., 1997), and selected a number of compounds for testing. Several series of active compounds were identified through the confirmation SEIA assay. One of these compounds is the ERI-7 series (Fig. 1 for structure and Fig. 3b for docking model). Of the 36 compounds with similar structures in this series that were tested, 14 showed over 80% maximal inhibition with IC50s ranging from 0.79 μM to 31 μM. As shown in Fig. 3c, ERI-7 was effective in inhibiting ERs interaction with all the SRC coactivators with similar potencies (IC50s were all around 25 μM). Interestingly, this compound seemed to be ERα selective since it did not disrupt ERβ or PR and SRC-3 interaction (Fig. 2c). Unfortunately, this series of compounds was not active in cell-based assays due to their low membrane permeability (data not shown).

3.3. ERI-5 and ERI-7 do not displace estradiol in ERα competition assay

We have shown above two new classes of ERs inhibitors with distinct chemical structures. Compounds from both series were able to inhibit ERs function by disrupting receptor/coactivator interaction. To further examine whether these compounds could bind to ERs directly and displace estradiol, we tested these ligands in an estrogen displacement assay using hERα LBD and radio-labeled 17β-estradiol. In this assay, unlabeled ligand, such as 17-β-estradiol, when added to the reaction, competes with 3[H]-17-β-estradiol for receptor binding, resulting in decreased binding of labeled ligand. As shown in Fig. 4, both ERI-5 and ERI-7 were not able to displace radio-labeled estradiol at concentrations up to 30 μM. The inability of these compounds to displace estradiol in this assay suggested that these compounds might bind poorly or not at all to the receptor. Alternatively, they might bind to the receptor at a novel binding site different from the estrogen binding site, and therefore were ineffective in displacing estradiol.

3.4. ERI-5 and ERI-7 bind to the estrogen receptor directly

Since ERI-5 and ERI-7 do not displace 17-β-estradiol as shown above, it is important to address whether they bind to the receptor directly. In an effort to verify that these inhibitors indeed bind directly to the ERs, we tested these compounds in a nuclear magnetic resonance assay whereby the ability of small molecules to bind ERs was monitored by one-dimensional NMR line-broadening experiments. The intrinsic line-width of a NMR spectrum is directly related to the molecular-weight of the molecule, where line-width increases with increasing molecular-weight (for review, see [20]). Therefore, observing an increase in the NMR line-width of a small molecular-weight compound upon the addition of ERs would be consistent with direct binding. As shown in Fig. 5, each free small molecule in the solution yielded a distinct peak pattern. Purified recombinant ERα LBD protein (pre-bound with an in house estrogen at 1:1 ratio) (see Section 2) was added to the solution in increasing amounts. In the case of 4-OHT, the peaks were broadened in the presence of the receptor in a dose dependent manner, suggesting a direct and stoichiometric binding of the receptor to 4-OHT. Meanwhile, small peaks corresponding to free estrogen molecules appeared (shaded), indicating bound estradiol was displaced by 4-OHT, and became free molecules in the solution. For compounds ERI-5 and ERI-7, line-broadening was observed dose dependently in both cases upon addition of ERα protein. However, no free estrogen peaks were seen. This experiment suggested that there was a direct binding between ERI-5 or ERI-7 and the ERα protein. Furthermore, these compounds, when bound to the receptor, did not displace receptor bound estradiol.
Fig. 2. (a) ERI-5 inhibited ERα/SRC interaction in mammalian two hybrid assay. COS7 cells cotransfected with ERαLBD and SRC-1 (△) or SRC-3 (△) were treated with ERI-5 at various concentrations in the presence of 1 nM 17β-estradiol (E₂). E₂ alone treatment was referred as the maximal activity (100%). Gal4DBD-VP16 treated with ERI-5 was used to detect cell toxicity as well as non-specific inhibition (△). ICI-control (▲) referred to the activity of ICI182,780 in this assay. (b) ERI-5 inhibited ERα/SRC interaction in SEIA assay. GST-ERαLBD and the NRID of SRC-1 (△), SRC-2 (△) or SRC-3 (△) recombinant proteins were used in this assay. Proteins were incubated with ERI-5 at various concentrations in the presence of 10 nM E₂. E₂ alone treatment was referred as the maximal activity (100%). ICI-control (▲) referred to the activity of ICI182,780 in this assay. (c) Cross activity of ERI-5 and ERI-7 on ERα and PR in SEIA assay. GST-ERαLBD (closed bar) or GST-PRLBD (open bar) and SRC-3 NRID were incubated with 10 nM E₂ or progesterone (P₄), respectively, alone or with 100 μM ERI-5 or ERI-7. Agonist alone treatment was referred as the maximal activity (100%). Data was analyzed by one-way ANOVA with LSD tests. **P-value <0.001 when compared to agonist alone treatment.

3.5. ERI-5 may induce a unique receptor conformation

Protease digestion assays have been used to detect ligand induced conformational changes for nuclear receptors. To examine whether these novel ER inhibitors were able to produce a different conformation on ERα, we carried out a partial enzymatic digestion assay. Full length ³⁵S labeled ERα was generated by in vitro transcription/translation in reticulocyte lysate and subjected to trypsin digestion (50 μg/ml) with different time treatments in the presence of various ligands. Peptides generated under conditions of limited proteolysis were resolved by gel electrophoresis. In the presence of 10 nM 17β-estradiol, a 35 kDa fragment (Fig. 6, arrow A) was generated with 5 min of trypsin
digested. This band appeared to be unique to the agonist treatment since it was not produced by the antagonist treatment. In the sample treated with 10 nM 4-OHT, a 28 kDa band (arrow B) appeared to be more resistant to the enzymatic digestion at the 5 min trypsin treatment when compared to the control and 17-estradiol treated samples. Samples treated with 10 μM ERI-7 produced a digestion pattern that was similar to that of the vehicle control, suggesting that it did not bind to the receptor, or the conformation generated could not be distinguished under the assay conditions. Samples treated with 10 μM ERI-5, however, generated a unique digestion pattern. Though overall its digestion pattern is similar to that of 4-OHT treatments, ERI-5 bound receptor appeared to be more stable against the enzymatic digestion. The receptor conformation seemed to be stabilized (band C) and not being further digested into smaller fragments (bands D and E) even after 20 min digestion. Without further analysis, it is not known whether the sustained fragment (band C) is the same as the fragment that migrates to the same position in other treatments. Nonetheless, this result suggested that ERI-5 might induce a receptor conformation that was different from other anti-estrogens. It also further confirmed that ERI-5 bound to the receptor directly.

3.6. ERI-5 inhibits endogenous Erα function in MCF-7 cells

We have identified compounds that were able to inhibit the ERα/SRC protein-protein interaction. These compounds appear to inhibit ERα function through a novel mechanism. Unlike the conventional antiestrogens, these inhibitors seem to bind to the ERα at a novel site that is different from the estrogen binding-pocket, and either directly or allosterically block the interaction with coactivator. Since all the

---

Fig. 3. (a) Ribbon diagram of the DES bound Erα LBD with a LxxLL peptide from Gsp1 NR-box II. (b) Diagram of the DES bound Erα LBD with a small molecule ERI-7 docking on the LxxLL peptide binding site. (c) ERI-7 inhibited Erα/SRC interaction induced by 17-estradiol in SEIA assay. GST-ErosLBD and NRID of SRC-1 (●), SRC-2 (∆) or SRC-3 (▼) were incubated with ERI-7 in the presence of 10 nM E2 at various concentrations. E2 alone treatment was referred as the maximal activity (100%). ICI-control (○) referred to the activity of ICI182,780 in this assay.
Gal4DBD-VP16, a control that can activate GRE-Luc con-
tact with 17-β-estradiol in the receptor competition assay. The cell extract containing ERα LBD protein was incubated with [3H] labeled 17-β-estradiol alone (referred as the maximal activity (100%), or with unlabeled ligands at various concentrations. The amount of [3H] labeled E2 used was determined by its EC50 in each ERα LBD protein preparation. In samples with unlabeled ligands, the remaining radio-labeled ligand bound to the receptor was measured by scintillation counter, and calculated as the percentage of maximum count.

experiments used in vitro systems with either truncated re-
ceptor or coactivator, it is important to demonstrate that these compounds can function against the endogenous re-
ceptor. To this purpose, we examined whether these com-
pounds could inhibit the expression of an ERα regulated gene pS2 in MCF-7 cells, a human breast cancer cell line. When MCF-7 cells were treated with 17-β-estradiol, pS2 gene transcription was upregulated as detected by real time quantitative PCR (Taqman) (Fig. 7, lane 2, [21]). This in-
duction was reversed by addition of an antiestrogen 4-OHT (Fig. 7, lane 3). ERI-5 could also inhibit estrogen induced pS2 gene transcription to some extent at concentrations of 10 μM and 20 μM (P-value < 0.001) in a dose dependent manner (P-value < 0.05). Cells were much less viable when treated with concentration higher than 20 μM (as indicated by GAPDH level, data not shown). The toxicity of the compound at higher concentration was also indicated in the M2H assay performed in COS7 cells. In COS7 cells, Gal4DBD-VP16, a control that can activate GRE-Luc con-
tact with 17-β-estradiol for recep-
tor binding (Fig. 4). The possible direct binding of these compounds to the receptor was also suggested by the NMR study (Fig. 5). Most of the compounds in these two series, however, could not be tested in cell based assays due to their low membrane permeability. The compound that was per-
méable in cells, ERI-5, did show inhibition of endogenous estrogen bound receptor outside of the ligand binding pocket, either directly or allosterically prevent SRC-3 from binding. This third type of inhibitor was also screened using a computer-based vir-
tual screen based on the crystal structure of the coactivator docking site of an agonist bound receptor. Based on crys-
tallography, small molecules that were able to dock to the LxxLL peptide binding site are likely to mimic the action of the peptide, therefore competing with the coactivator for receptor binding.

We report here the characterization of novel ERα antago-
nists that appear to bind to ERα through a novel binding site(s). We have shown that ERI-5 and ERI-7 can inhibit ERαs and p160 coactivator interaction. Unlike other known ERα antagonists, however, these compounds do not displace 17-β-estradiol in the receptor competition assay, implying that they may bind to a different binding site on the receptor, hence they do not compete with 17-β-estradiol for recep-
tor binding (Fig. 4). The possible direct binding of these compounds to the receptor was also suggested by the NMR study (Fig. 5). Most of the compounds in these two series, however, could not be tested in cell based assays due to their low membrane permeability. The compound that was per-
méable in cells, ERI-5, did show inhibition of endogenous ERα transactivation function in MCF7 cells. These data sug-
gest that these compounds are bona fide ERα inhibitors that recognize the ERαs and inhibit the receptor function through disrupting coactivator recruitment. It is well established that classical steroid receptor antag-
ons bind to the receptor and induce a conformational change that is different from the agonist bound receptor [22]. The change is mainly in the AF2 helix position, which dictates the inter-
action with coactivators. As suggested by X-ray crystallog-
raphy, the AF2 helix in the apo-receptor or antagonist-bound receptor extends downward away from the body of the LBD. In contrast, all of the agonist-bound structures have the AF2 helix packed against the body of the LBD, forming an...
essential part of the charge-clamp for coactivator binding 
[22,23]. This conformational change is also reflected in the 
partial proteolytic digestion assay. Trypsin digestion of ag-
onist bound receptor yields a protected band that is not seen 
with the antagonist bound receptor, presumably due to AF2 
protection. Our novel ERα inhibitor ERI-5 did not yield this 
agonist-protected band in the tryptic digestion assay. This 
data suggests that ERI-5 may block the recruitment of coac-
tivator mainly by affecting the AF2 position as well.

The identification of these novel ERα antagonists further 
demonstrates that coactivator interaction is essential for 
receptor function. Though these compounds are far from 
being drug candidates, improved compounds with a sim-
ilar mechanism of action may provide an alternative for 
the current antiestrogen therapy. Since these compounds 
do not compete with estrogen, the treatment would not 
generate free estrogen in the body, which may cross react 
with other biological pathways. Furthermore, by function-
Fig. 6. Partial proteolytic digestion of ERI-5 and ERI-7 bound ER/H9251. Full length 35 S labeled ER/H9251 generated by in vitro transcription/translation (input) was incubated with ligands (10 nM 17-estradiol or 4-OHT, 10 μM ERI-5, or ERI-7) for 1 h at room temperature prior to trypsin digestion (50 μg/ml) for various time points (5, 10, and 20 min). Fragmented receptor was resolved on SDS-PAGE gel.

Fig. 7. ERI-5 inhibited endogenous ER/H9251 function in MCF-7 cells. MCF-7 cells were treated with either 1 nM 17-estradiol alone, or with 4-OHT (1 μM) or ERI-5 (10 and 20 μM) for overnight. Total RNA were collected and subjected to real time quantitative RT-PCR (Taqman, ABI) to detect the mRNA level of pS2 gene. GAPDH was used as internal control. Quantitated pS2 mRNA was normalized by the GAPDH mRNA. One-way ANOVA with LSD test was performed to analyzed the data. **P-value < 0.001 when compared to lane 2. *P-value < 0.05 when compared to lane 4.

References


Acknowledgements

Authors thank Ms. Jennifer Bray for the cell culture work, Dr. Desiree Tsao for the technical support in NMR, Drs. Zhiming Zhang and Jeffery Bray for helpful discussion and advice.


