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Identification of novel estrogen receptor α antagonists

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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 17-β-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 17-β-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.

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 ERα 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 coativator 1 (SRC-1/NcoA1) [2], SRC-2/GRIP1/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.

ERα plays important roles in diverse physiological pathways. Estradiol and compounds that modulate ERα 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 ERα 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

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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 17-β-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 pCDNA3.1 (Invitrogen) along with a GAL4 responsive luciferase (GRE-Luc) reporter. The endogenous transcription activation function of SRC-3 was exploited instead of using a fusion with the VP16 activation domain (AD). A control assay consisted of the same GRE-Luc transfected along with a one-hybrid fusion of GAL4 DBD and VP16AD (pM3VP16, Clontech).

In advance of high-throughput screening (HTS), batches of 1×10^9 COS-7 cells were transfected with two hybrid plasmids in suspension using Lipofectamine 2000 (Invitrogen) and aliquots were frozen in media containing 5% DMSO (dimethysulfoxide). For HTS, cells were thawed and plated at 2400 cells per well in 384 well plates. Cells were treated with 3μg/ml test compound in the presence of 1 nM 17-β-estradiol for 16 h. Luciferase activity was measured on a TopCount (Packard) luminometer using LucScreen reagent (Tropix) as recommended by the manufacturer. Active samples were defined as those that showed a signal reduction of 50% or more in duplicate assays. These samples were then confirmed using freshly transfected cells in parallel with the control assay to eliminate false positives.

2.2. SRC/ERα interaction analysis (SEIA)

FLAG-tagged ERα was produced in a bacculovirus 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).

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].

2.4. 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 OD_{600} =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

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 ERα containing lysate for the binding assay was determined for each preparation, and dilution was made accordingly in the assay buffer [1 mM EDTA in 1X DPBS (without Ca^{2+} and Mg^{2+})]. 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 ^{3}H-estradiol (2 nM 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 (17-β-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 ERα 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 cyroprobe. The 1D NMR spectra were collected with a sweep-width of 8992.8 Hz with 16 K 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): ^{35}S labeled ERα was produced in rabbit reticulocyte lysate using the TNT coupled transcription/translation system (Promega). The ERα receptor was incubated with ligands (10 nM 17-β-estradiol or 4-OHT, or 10 μM cpds) 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 ERα LBD interacts with a number of coactivators including SRC-3. This receptor/coactivator interaction is essential for ERα 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 17-β-estradiol, ERα LBD will interact with SRC-3, leading to the activation of the reporter gene. Addition of an excess amount of ERα antagonist, 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 17-β-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 ERα 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 ERα LBD and SRC NRID (see Section 2) (Fig. 2b). The compound also well antagonized the ability of ERβ, 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 ERα 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

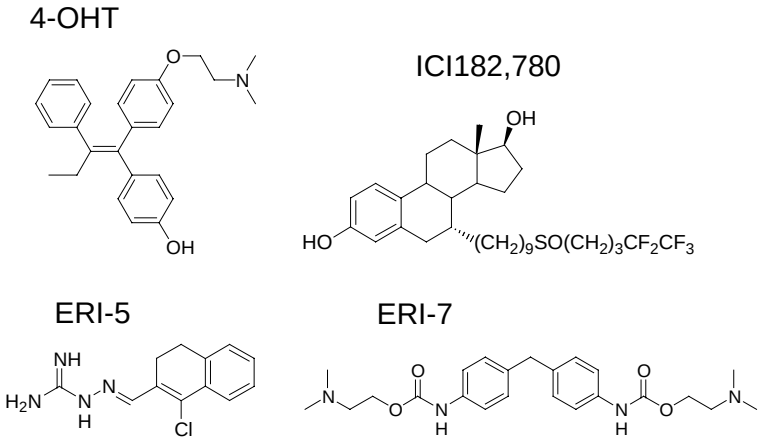

Fig. 1. Chemical structures of estrogen receptor antagonists.

contacting the ERα coactivator interaction surface (Fig. 3b). This would permit us to identify novel ERα 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 ERα 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 ERα inhibitors with distinct chemical structures. Compounds from both series were able to inhibit ERα function by disrupting receptor/coactivator interaction. To further examine whether these compounds could bind to ERα directly and displace estradiol, we tested these ligands in an estrogen displacement assay using hERα LBD and radio-labeled 17-β-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 estrogen 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 ERα, we tested these compounds in a nuclear magnetic resonance assay whereby the ability of small molecules to bind ERα 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 ERα 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.

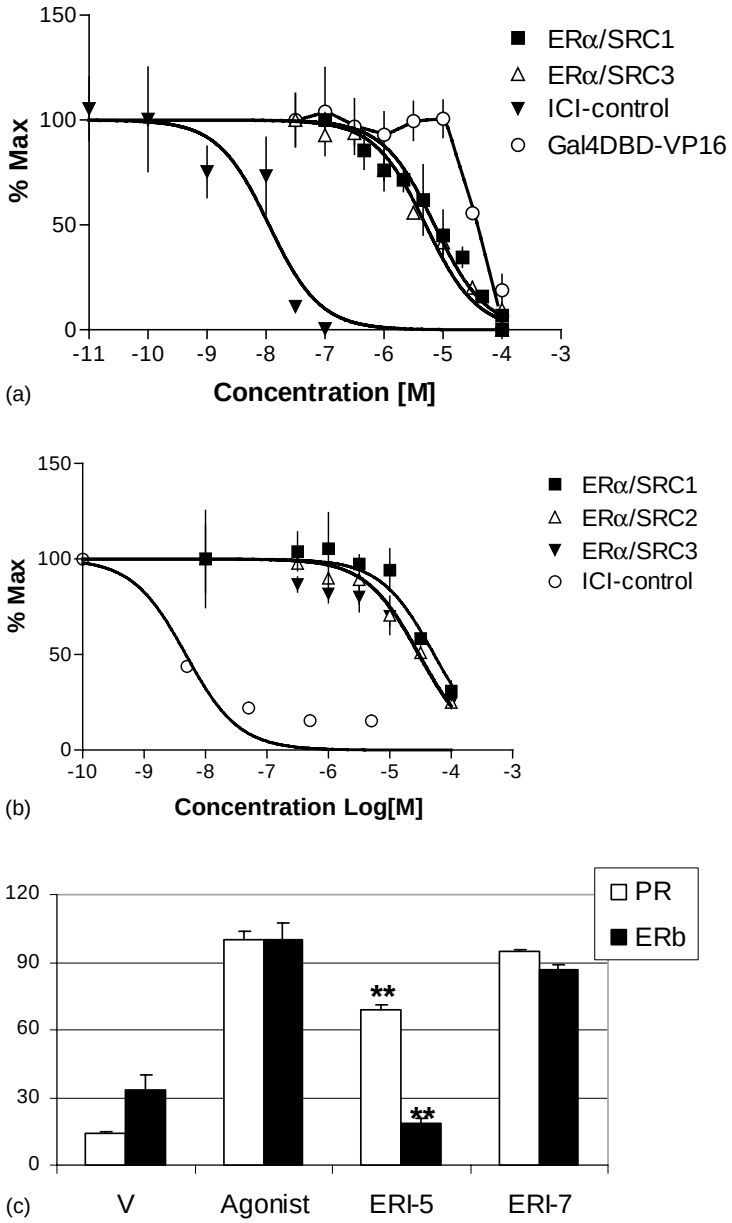

Fig. 2. (a) ERI-5 inhibited ERα/SRC interaction in mammalian two hybrid assay. COS7 cells cotransfected with ERαLBD and SRC-1 (■) or SRC-3 (△) were treated with ERI-5 at various concentrations in the presence of 1 nM 17-β-estradiol (E_2). E_2 alone treatment was referred as the maximal activity (100%). 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 E_2. E_2 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 ERβ and PR in SEIA assay. GST-ERβLBD (closed bar) or GST-PRLBD (open bar) and SRC-3 NRID were incubated with 10 nM E_2 or progesterone (P_4), 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 ^{35}S 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 17-β-estradiol, a 35 kDa fragment (Fig. 6, arrow A) was generated with 5 min of trypsin

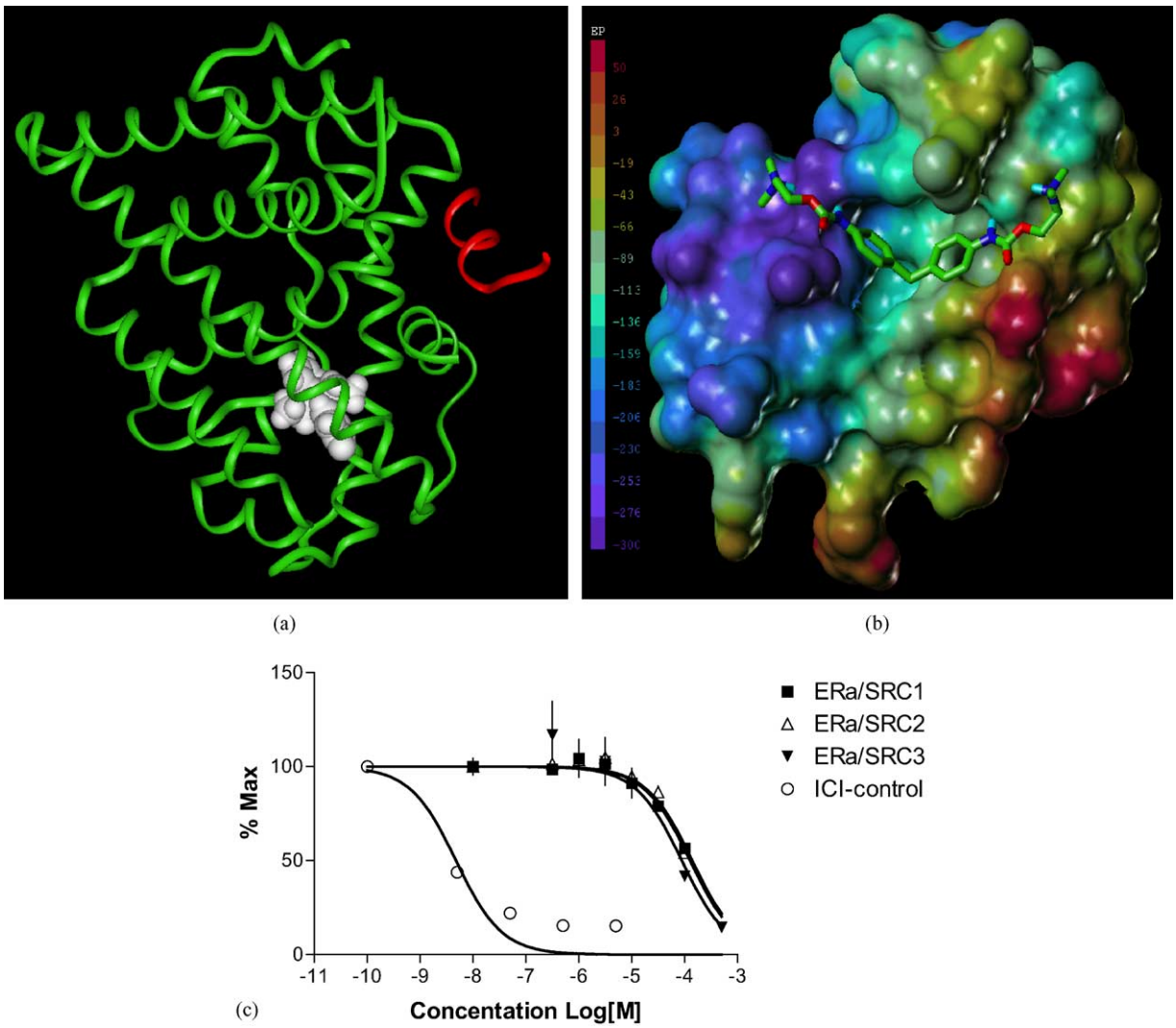

Fig. 3. (a) Ribbon diagram of the DES bound ERα LBD with a LxxLL peptide from Grip1 NR-box II. (b) Diagram of the DES bound ERα LBD with a small molecule ERI-7 docking on the LxxLL peptide binding site. (c) ERI-7 inhibited ERα/SRC interaction induced by 17-β-estradiol in SEIA assay. GST-ERαLBD and NRID of SRC-1 (■), SRC-2 (△) or SRC-3 (▼) were incubated with ERI-7 in the presence of 10 nM E_2 at various concentrations. E_2 alone treatment was referred as the maximal activity (100%). ICI-control (○) referred to the activity of ICI182,780 in this assay.

digestion. 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-β-estradiol treated samples. Samples treated with 10 μM ERI-7 produced a digestion pattern that was similar to that of the vehicle control, suggesting either 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

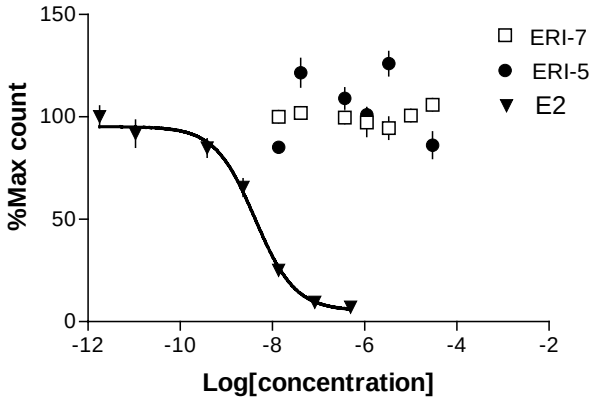

Fig. 4. ERI-5 and ERI-7 did not compete with 17-β-estradiol in the estrogen receptor competition assay. *E. coli* extract containing ERα LBD protein was incubated with ^{3}[H] labeled 17-β-estradiol alone [referred as the maximal activity (100%)], or with unlabeled ligands at various concentrations. The amount of ^{3}[H] labeled E_2 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 receptor or coactivator, it is important to demonstrate that these compounds can function against the endogenous receptor. To this purpose, we examined whether these compounds 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 induction 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 constitutively, showed a near 50% decrease in activity at 30 μM (Fig. 2a, Gal4DBD-VP16 control). Therefore, it is not advisable to treat cells with higher concentration to achieve maximal inhibition with this compound. Nonetheless, this experiment suggested that although it showed low potency, ERI-5 could inhibit the endogenous ERα function in MCF7 cells. Compounds generated from the virtual screen, such as ERI-7 could not be evaluated in this assay due to low membrane permeability (data not shown).

4. Discussion

Estrogen receptors, like many other nuclear receptors, require coactivators in order to transactivate their target genes [1]. The ability of ERα to interact with coactivators is manifested by ligands that bind to the LBD ligand-binding pocket. Upon agonist binding, the receptor undergoes conformational changes, forming a hydrophobic interaction interface in the LBD region that provides a docking site for the coactivators [11]. An antagonist, on the other hand, prevents the formation of such a docking site, therefore, blocking the recruitment of coactivators [22]. We developed a modified mammalian two-hybrid assay using Gal4DBD-ERαLBD and full length SRC-3. Estrogen bound ERαLBD would recruit SRC-3 and subsequently activate transcription of a reporter gene containing a Gal4 response element. Compounds that inhibited transactivation were selected for further analysis. The inhibition can be achieved by three classes of inhibitors through different mechanisms. These inhibitors could be: (1) classical antiestrogens that compete with estrogen binding, and prohibit the association of coactivator; (2) compounds that inhibit SRC-3 activity by either disrupting receptor interaction or transactivation function; and (3) small molecules that bind to the 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 virtual screen based on the crystal structure of the coactivator docking site of an agonist bound receptor. Based on crystallography, 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 identification of novel ERα antagonists that appear to bind to ERα through a novel binding site(s). We have shown that ERI-5 and ERI-7 can inhibit ERα and p160 coactivator interaction. Unlike other known ERα antagonists, however, these compounds do not displace 17-β-estradiol in the receptor competition assay, implying they may bind to a different binding site on the receptor, hence they do not compete with 17-β-estradiol for receptor 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 permeable in cells, ERI-5, did show inhibition of endogenous ERα transactivation function in MCF7 cells. These data suggest that these compounds are bona fide ERα inhibitors that recognize the ERα and inhibit the receptor function through disrupting coactivator recruitment.

It is well established that classical steroid receptor antagonists bind to the receptor and induce a conformation that is different from the agonist bound receptor [22]. The change is mainly in the AF2 helix position, which dictates the interaction with coactivators. As suggested by X-ray crystallography, 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

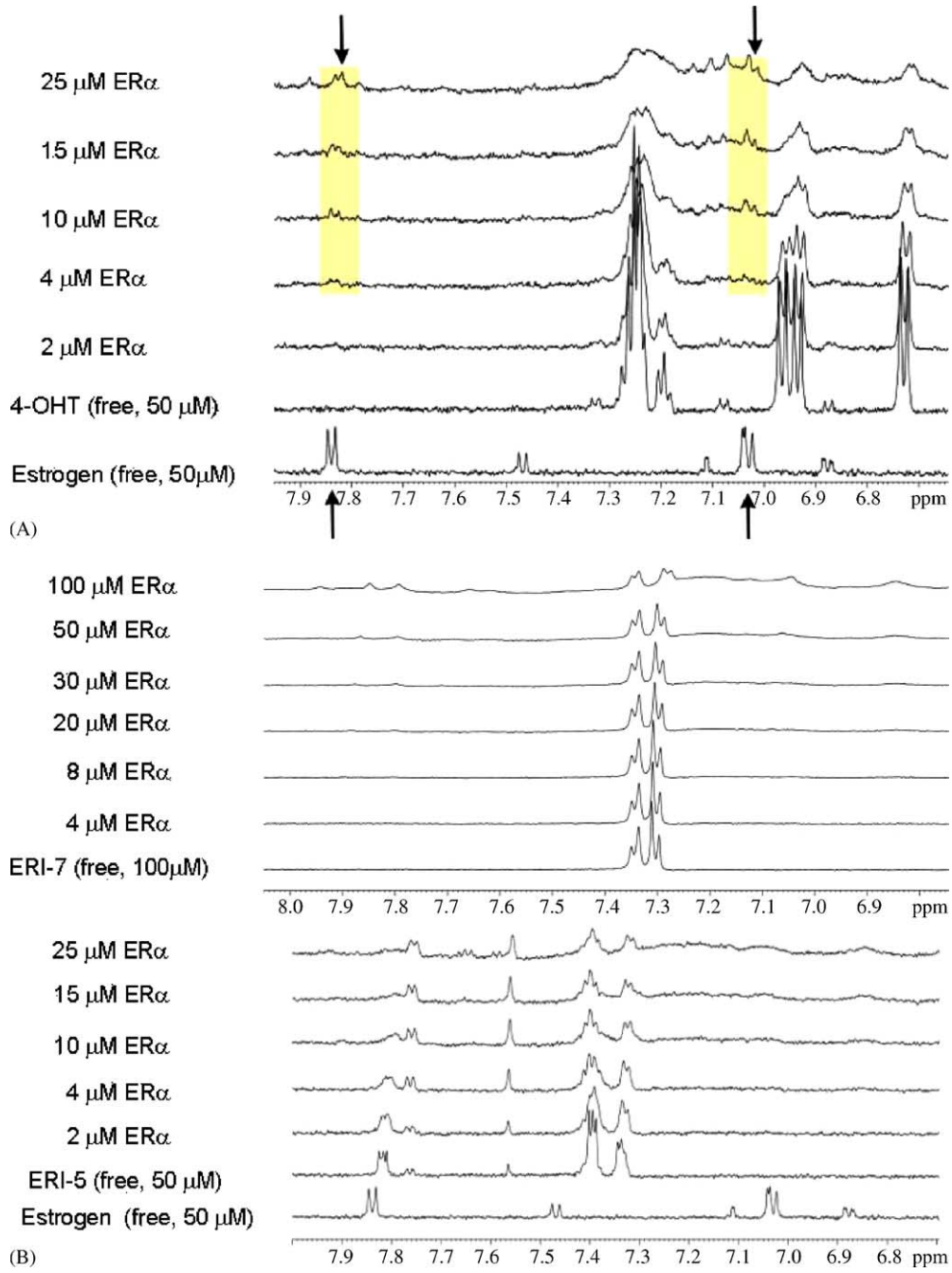

Fig. 5. ERI-5 and ERI-7 bound to ERα protein directly in NMR assay. The spectra of free 4-OHT, ERI-7, ERI-5 and estrogen in solution are shown. Solutions containing free compounds were titrated with increasing amount of ERα LBD protein pre-bound with estrogen in 1:1 ratio. The spectrum was recorded at each titration point. (A) 4-OHT bound to the ERα LBD, and displaced estrogen. Free estrogen peaks are shown in shades. (B) ERI-7 and ERI-5 bound to the ERα LBD, but no estrogen displacement occurred.

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 agonist 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 coactivator 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 similar 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-

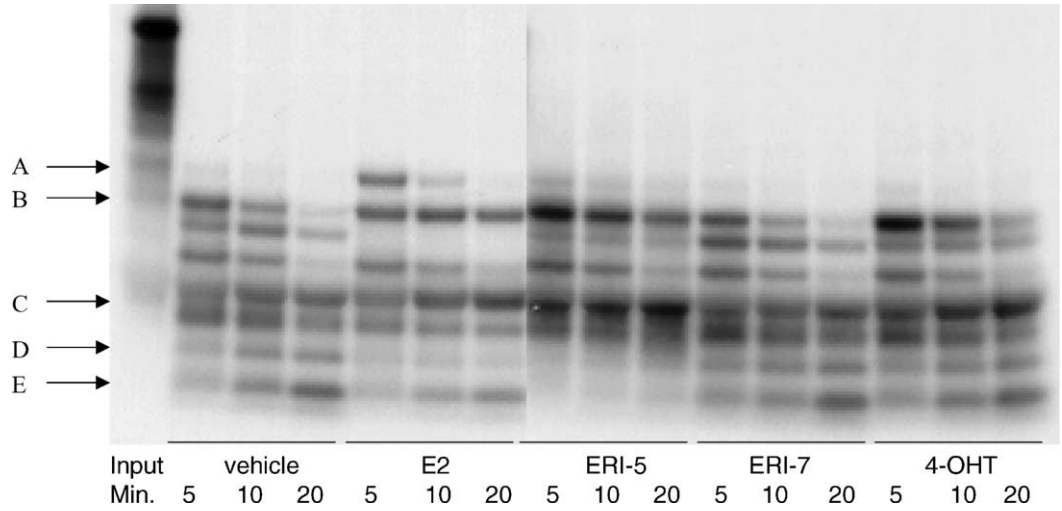

Fig. 6. Partial proteolytic digestion of ERI-5 and ERI-7 bound ERα. Full length ^{35}S labeled ERα generated by in vitro transcription/translation (input) was incubated with ligands (10 nM 17-β-estradiol or 4-OHT, 10 μM ERI-5, or ERI-7) for 1 h at room temperature prior to trypsin digestion (50 μg/ml) for various time points (5, 10, and 20 min). Fragmented receptor was resolved on SDS-PAGE gel.

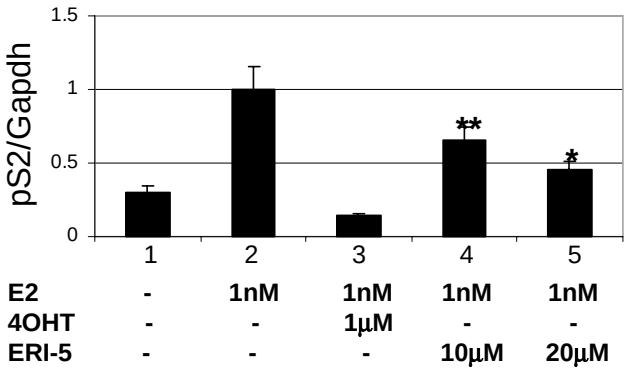

Fig. 7. ERI-5 inhibited endogenous ERα function in MCF-7 cells. MCF-7 cells were treated with either 1 nM 17-β-estradiol alone, or with 4-OHT (1 μM) or ERI-5 (10 and 20 μM) for overnight. Total RNA were collected and subjected to real time quantitative RT-PCR (Taqman, ABI) to detect the mRNA level of *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.

ing through a different mechanism, these small molecules might not be compromised by the development of hormonal resistance often seen in long term antiestrogen therapy [12].

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