Definition of Functionally Important Mechanistic Differences among Selective Estrogen Receptor Down-regulators

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Abstract

One subclass of antiestrogens, the selective estrogen receptor down-regulators (SERDs), have received considerable attention in recent years due to their potential role in the management of breast cancer. These compounds are designed to competitively bind to estrogen receptors (ERs), leading to a rapid, proteasome-dependent degradation of the receptor. Contained within this class of molecules is the steroidal antiestrogen ICI182,780 (faslodex), recently approved for the treatment of metastatic cancer, and GW5638/DPC974, a SERD that is currently being evaluated in the clinic. Given that mechanistic differences between different selective estrogen receptor modulators have been translated into important clinical profiles, it was of interest to determine if the SERD subclass of ligands were likewise functionally or mechanistically distinguishable. In this study, we show that although the steroidal and nonsteroidal SERDs target ER\alpha for degradation, the underlying mechanism(s) are different. Of note was the identification of a specific protein-protein interaction surface presented on ER\alpha in the presence of the ICI182,780-activated receptor which is required for degradation. Interestingly, this surface is also present on ER\alpha in the presence of RU58,668, a SERD that is chemically distinct from ICI182,780. This surface is not required for GW5638-mediated degradation, and thus, this SERD seems to affect ER\alpha down-regulation by a different mechanism. These data suggest that sequencing of therapies using drugs of this class is likely to be possible. Finally, because of the unmet need for orally active SERDS that function similarly to ICI182,780, we have used the insights from these mechanistic studies to develop and validate a high-throughput screen for compounds of this class with improved pharmaceutical properties.

Introduction

The biological actions of estrogens are manifested through two high-affinity estrogen receptors, ER\alpha and ER\beta, which are expressed at different levels in target cells (1–3). In the absence of their cognate hormones, these receptors reside in the cytoplasm or nuclei of target cells associated with an inhibitory heat-shock protein complex (4). After binding estrogen, they undergo activating conformational changes that lead to the displacement of heat-shock proteins, an event that facilitates the formation of receptor homodimers or heterodimers and their subsequent interaction with specific DNA response elements within the regulatory regions of target genes. The DNA-bound receptor can then either positively or negatively regulate target gene transcription. Alternate non-genomic pathways by which estrogen-activated ERs or ER\beta can activate signaling processes at the membrane or in the cytoplasm have been identified, although the physiologic significance of these responses remains controversial (5).

Although generally considered a reproductive hormone, the expression of these ERs in nonreproductive tissues indicates that the regulatory actions of estrogens extend beyond reproduction (6, 7). Not surprisingly, therefore, pharmaceuticals that both positively and negatively regulate ER action are widely used in the clinic. Outside the realm of normal physiology, however, it has been shown that the majority of breast tumors express ERs and are responsive to the mitogenic actions of estrogen(s) (ref. 8). Furthermore, it has been shown that signaling pathways, initiated at the cell membrane, can impinge on ERs positively, regulating its activity in the presence or absence of exogenous estrogens (9). A protective role of ER\beta in breast cancer cells has been inferred from studies done in vitro, although its role(s) in tumor pathology has not been determined (10–12). Regardless, the clinical benefit of disrupting estrogen signaling in the breast using antiestrogens, compounds that competitively inhibit estrogen binding to ERs, or aromatase inhibitors, compounds that block estrogen production, are well established (13). Although it has recently become clear that aromatase inhibitors are superior to antiestrogens as first-line hormonal interventions in metastatic breast cancer, the chronic nature of the disease makes it likely that both types of drugs will play a role in the management of this disease (13–15). Not surprisingly, therefore, a considerable amount of attention is now being paid to the development of different sequencing regimens that may provide increased therapeutic benefit. Therefore, interest in identifying new antiestrogens with unique mechanisms of action has increased considerably over the past few years.

The currently available antiestrogens fall into two general classes (a) selective estrogen receptor modulators (SERMs), such as tamoxifen, and (b) selective estrogen receptor down-regulators (SERDs), such as the pure antiestrogen faslodex (IC1182,780; ref. 13). Tamoxifen functions by competitively displacing estradiol from the receptor, disrupting the primary coactivator-binding pocket on the receptor and blocking the access of ER, activated by residual estrogens, to its target gene promoters. The SERDs share these attributes with tamoxifen but, in addition, they induce a rapid and quantitative down-regulation of ERs (14, 16). It is likely that it is the latter activity which is responsible for the dramatic results observed in patients treated with faslodex who have failed at least one endocrine therapy and which has made it a first-in-class SERD approved for the treatment of metastatic breast cancer (16–19). Although clinical studies have shown some success with faslodex, its poor pharmacodynamic properties and lack of oral bioavailability have limited its clinical utility (16–20). Thus, although faslodex has provided very important proof-of-concept information, it is clear that there is an unmet medical need for (a)}
faslodex-like compounds with improved pharmaceutical properties and (b) other SERDs/antiestrogens with distinct mechanisms of action. The rationale for the continued development of novel classes of ER antagonists has been detailed recently in several comprehensive reviews (21, 22). For this reason, we have undertaken a comparative study of the mechanism of action of faslodex and that of GW5638/DPC974, a chemically distinct SERD with a view to developing mechanism-based screens that will allow the identification of new antiestrogens (23–25).

Materials and Methods

Plasmids, chemicals, and antibodies. Plasmids were previously described (26–28) or constructed through site-directed mutagenesis (QuickChange kit; Stratagene), PCR amplification, restriction enzyme subcloning, and/or “Gateway” LR reactions (Invitrogen). More detailed instructions on plasmid construction are available on request. 17β-Estradiol (E2), 4-hydroxy-tamoxifen (TOT), dexamethasone, and 9-cis-retinoic acid were purchased from Sigma. Raklofoxime, EM-652, GW7604, RU58-668, and RU486 were provided as gifts from Dr. E. Larson (Pfizer, Inc., Groton, CT), Dr. Fernand Labrie (CHUL Research Center, Quebec City, Quebec, Canada), Dr. T. Willson (GlaxoSmithKline, Research Triangle Park, NC), Michel Renoir (UMR Centre National de la Recherche Scientifique, France), and Lindag Pharmaceuticals, Inc. (San Diego, CA), respectively. ICI182,780 was obtained from Tocris. Casodex was provided by GlaxoSmithKline. R1881 and R5020 were purchased from NEN Life Science Products. Antibodies used included EBOX, lamin a, α-tubulin, cytokeratin 18, and GAL4-DBD antibody (Santa Cruz Biotechnology), V5 (Invitrogen), and anti–enhanced green fluorescent protein (EGFP) polyclonal antibody (Clontech).

ERα protein and RNA levels. MCF7 cells were maintained in DMEM/F12 (Invitrogen) supplemented with 10% fetal bovine serum (HyClone), 0.1 mmol/L of nonessential amino acids, and 1 mmol/L of sodium pyruvate (Invitrogen). MCF7 cells were plated in six-well dishes at 500,000 cells per well containing phenol red–free DMEM/F12 supplemented with 5% charcoal-stripped fetal bovine serum for 36 h. Cells were subsequently treated with either vehicle or 100 nmol/L of ERα ligand for the times indicated in Fig. 1. Whole cell lysates were harvested by boiling the cells in Laemml buffer and were subjected to SDS-PAGE and Western analysis. Following ligand treatments, ERα mRNA levels were detected by quantitative PCR using primers specific to ERα and normalized to 36B4 mRNA levels.

Cell fractionation and analysis. MCF7 cells were plated as previously described and subsequently treated with either vehicle, 100 nmol/L of E2, ICI182,780, or GW7604 for the times specified in Fig. 2. Cells were harvested, and cell pellets were resuspended on ice for 15 min in 100 μL of cytoplasmic extraction buffer [10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA] containing protease inhibitor cocktail set III (Calbiochem). Cells were permeabilized by adding IGEPA (Sigma) to 0.63% total volume, vortexed for 10 s, and centrifuged for 1 min to obtain the cytoplasmic extract. To obtain soluble nuclear extracts, nuclear pellets were resuspended on ice for 20 min in 100 μL of nuclear extract buffer (20 mmol/L HEPES, 25% total volume glycerol, 0.4 mol/L NaCl, 1 mmol/L EDTA, and 1 mmol/L EGTA) with protease inhibitors, and centrifuged for 5 min. The remaining pellets were sequentially extracted in nuclear matrix digestion buffer containing protease inhibitors and DNase 1 (Roche), 0.25 mol/L of ammonium sulfate, and finally, 2.0 mol/L of NaCl as previously described (29), and combined to a total volume of 100 μL to compose the detergent-extractable nuclear matrix fraction. The resulting insoluble fraction was solubilized in 100 μL of 95% Laemml buffer (Bio-Rad) with 5% β-mercaptoethanol and boiled at 95°C for 20 min. The protein concentration of the cytoplasmic extracts was determined and the volume constituting 100 μg of cytoplasmic extract was analyzed by SDS-PAGE. In addition, equivalent volumes of the nuclear extract, detergent-extractable nuclear matrix, and insoluble fractions were loaded. Proteins were transferred to nitrocellulose membrane and subjected to Western analysis.

T7 phage display. mRNA was obtained from HepG2, MCF7, and HEC1B cells using the Straight A’s mRNA kit (Novagen). T7 phage libraries were constructed using OrientExpress cDNA kit and T7 Select kit according to the instructions of the manufacturer (Novagen). Costar 96-well plates were coated with 20 μg of neutravidin in 100 μL of 100 mmol/L NaHCO3 overnight at 4°C. Plates were blocked with 150 μL of 2% milk in 100 mmol/L of NaHCO3 for 1 h at room temperature, then washed five times with PBST (PBST + 0.1% Tween 20). Two picomoles of biotinylated double-stranded DNA composed of the vitellogenin estrogen receptor response element in PBST were applied to each well for 1 h at room temperature and excess neutravidin was subsequently blocked for another hour with 10 μL of 1 mmol/L of biotin. Wells were washed five times with PBST and ~0.25 μg of baculovirus-expressed recombinant ERα (Affinity Bioreagents) in 100 μL of PBST containing vehicle or 1 μmol/L of ERα ligand was added to each well and incubated overnight at 4°C. After washing, wells were blocked with 150 μL of 2% milk in PBST for 1 h at room temperature, washed, and subsequently incubated with 1 × 108 plaque-forming units of T7 library in 100 μL of PBST containing vehicle or 1 μmol/L of ERα ligand for 1 h at room temperature. Following five washes with PBST, bound phages were eluted in a DNase elution mix (99 μL PBST, 0.5 μL of 1 mol/L MgCl2, and 0.5 μL of 10 mg/mL DNase I) for 1 h at room temperature. Eluted phages were amplified in a 10 mL culture of Escherichia coli BL21DE3 cells induced with 1 mmol/L of isopropyl-β-D-thiogalactopyranoside until complete bacterial lysis occurred. Amplified phages were harvested following centrifugation and a new plate containing ERα. After five rounds of panning, phage ELISAs were done to verify affinity enrichment of ERα-interacting phages. Specifically, amplified phages (100 μL/well) were added to wells containing ERα for 1 h at room temperature. Unbound phages were washed away and wells were incubated with 150 μL of a 1:5,000 dilution of anti–T7-HRP (Novagen) in PBST for 1 h at room temperature. After thoroughly washing the wells, bound phages were detected by incubating the wells with 100 μL of 2’-2’-azoni-bis-ethylenbenzthiazoline-6-sulfonic acid in the presence of 0.05% H2O2 and the color change was measured at 405 mmol/L with a plate reader (Multiskan MS, Labsystems). T7 phages from the rounds with the highest enrichment of ERα-interacting phages were plaque-purified and clones were randomly selected for PCR amplification of the cDNA inserts using T7 select UP and DOWN primer (Novagen). The inserts were sequenced, translated, and corresponding proteins were identified using the BLASTp program (National Center for Biotechnology Information).

Mammalian two-hybrid assays. HepG2 cells were maintained, plated, and transfected and mammalian two-hybrid assays were done as previously described (27).

ERα mutant degradation analysis. HeLa cells were maintained as previously described (30). For transient transfection and Western analysis of ERα, ERα mutants, and ERβ, HeLa cells were plated in six-well dishes containing phenol red–free media supplemented with 8% charcoal-stripped fetal bovine serum at 500,000 cells per well for 24 h. Cells were subsequently transfected with 1.5 μg of pEGFP, 0.3 μg of CMV-β-gal, and 1.5 μg of pCDNA 3.1-V5-ERα, -ERα mutant, or ERβ using Fugene (Roche) according to the instructions of the manufacturer for 24 h, and subsequently treated with vehicle or 100 nmol/L of ICI182,780 for 3 h. HeLa cells were harvested, and cell pellets were resuspended and mechanically disrupted by pipetting in low salt/low detergent radioimmunoprecipitation assay buffer [100 mmol/L Tris (pH 8.3), 2 mmol/L EDTA, 0.02% SDS, 0.5% IGEPA, and 150 mmol/L NaCl] containing protease inhibitors, and incubating on ice for 10 min. Soluble fractions were harvested and insoluble solubilized in an equivalent amount of Laemll buffer as previously described. Soluble protein lysates were subjected to β-gal assays, and protein volumes were loaded based on β-gal expression and subjected to Western analysis as previously described.

Antiestrogen-interacting peptide and control adenovirus construction and expression. GAL4-DBD–antiestrogen interacting peptide (AEP) and GAL4-DBD-control (AEP mutant within the ERα-binding region) adenoviruses were generated using the “Gateway” ViraPower Adenoviral Expression System according to the manufacturer’s protocol (Invitrogen) and purified using the Vira Bind Adenovirus Purification Kit according to the manufacturer’s instructions (Cell BioLabs, Inc.).
Figure 1. Estradiol, GW7604 and ICI182,780 induce a rapid and sustained decrease in ERα protein. A, compound structures of ERα ligands (21). MCF7 cells were grown as described in Materials and Methods and treated with either vehicle, or 100 nmol/L of 17\(^\beta\)-estradiol (E2), 4-hydroxy-tamoxifen (TOT), GW7604 (GW), raloxifene (Ral), EM-652 (EM), or ICI182,780 (ICI) for either 4 h (B) or 48 h (C). Whole cell extracts were obtained by boiling cells in Laemmli buffer and were subjected to Western analysis to detect ERα levels. Equal loading was determined by detection of \(\alpha\)-tubulin and cytokeratin 18. The percentage of remaining ERα was determined by densitometry readings using Image J and comparing ERα density to cytokeratin 18 density from two independent experiments for each treatment.

ERα RNA levels following 4 h (B) or 48 h (C) of treatment were detected by quantitative PCR and normalized to 36B4 mRNA levels. Columns, means from one of two independent experiments for each treatment.

Mechanism-Based Distinction of SERDs

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To measure the effects of adenovirally expressed AEIP on ERα translocation, MCF7 cells were plated at 500,000 cells per well in six-well dishes as previously described for 24 to 48 h. Cells were incubated with either pAD-DEST-GAL4-DBD-AEIP or -control virus at a multiplicity of infection of 150 in 50 mL of media for 2 h at room temperature. Following the 2 h incubation, 2 mL of media were added to the cells and the cells were returned to 37°C. Forty-eight hours later, the cells were treated with vehicle or 100 nmol/L of E2, ICI182,780, or GW7604 for the specified time periods. Cells were harvested and subjected to low salt/low detergent radioimmunoprecipitation assay buffer–mediated cell lysis. Soluble and insoluble extracts were subjected to Western analysis as previously described.

Results

There is a high level of interest in developing SERDs, a subclass of antiestrogens that both disrupt ERα-cofactor interactions and induce receptor turnover in cells. This interest is driven primarily by the observation that a compound of this pharmacologic class, ICI182,780 (faslodex), has shown remarkable efficacy as a second-line treatment for tamoxifen-resistant breast cancers (17). Little is known, however, about the mechanism(s) by which ERα degradation is accomplished upon interacting with SERDs, an impediment to the development of molecules with improved therapeutic properties. Thus, the primary objective of this study was to define the characteristics of compounds that enable them to induce ERα turnover and determine if chemically distinct SERDs function in a similar manner. As a first step in addressing this issue, we examined the stability of endogenous ERα in MCF-7 cells following treatment with either estradiol or selected SERMs and SERDs (Fig. 1 A). Specifically, total ERα protein and mRNA expression levels were examined in cells following acute (4 h) or chronic (48 h) treatments with each compound (Fig. 1 B and C). Western immunoblots and quantitative densitometry were used to measure ERα protein levels. Not surprisingly, no ERα protein was recovered from cells following 4 h of treatment with estradiol (Fig. 1 B). Unexpectedly, with the exception of TOT, all of the other ligands tested reduced ERα levels in cells albeit with different efficacies and these changes could not be attributed to changes in ERα mRNA levels. Most notably, ICI182,780 and the higher affinity 4-hydroxylated metabolite of GW5638 (GW7604) were as effective as estradiol in this acute experiment. No additional down-regulation of ERα protein was observed following extended (48 h) treatment with raloxifene or EM652 (Fig. 1 C). Western immunoblots and quantitative densitometry were used to measure ERα protein levels. Not surprisingly, no ERα protein was recovered from cells following 4 h of treatment with estradiol (Fig. 1 B). Unexpectedly, with the exception of TOT, all of the other ligands tested reduced ERα levels in cells albeit with different efficacies and these changes could not be attributed to changes in ERα mRNA levels. Most notably, ICI182,780 and the higher affinity 4-hydroxylated metabolite of GW5638 (GW7604) were as effective as estradiol in this acute experiment. No additional down-regulation of ERα protein was observed following extended (48 h) treatment with raloxifene or EM652 (Fig. 1 C). However, a significant increase in ERα levels was observed when TOT-treated cells were examined at this time point. The reappearance of ERα in the cells

Figure 2. Subcellular compartmentalization of ERα upon binding ICI182,780 is distinct from that observed in the presence of GW7604 and E2. MCF7 cells were grown as described in Materials and Methods and treated with either vehicle, 100 nmol/L of E2, 100 nmol/L of ICI182,780, or 100 nmol/L of GW7604 for the time periods indicated. Following treatment, the cells were harvested and lysed to obtain separate soluble cytoplasmic fractions (C), soluble nuclear fractions (N), soluble DNase/0.25 mol/L ammonium sulfate/2 mol/L NaCl fractions (D), and remaining insoluble fractions (I) as described in Materials and Methods. Fractions were subjected to Western analysis to detect ERα, α-tubulin (C fraction control), lamin A (N, D, I fraction controls), and cytokeratin 18 (loading and C and I fraction controls).
treated for 48 h suggests that, despite the presence of saturating amounts of estradiol, constitutive suppression of ERα levels by this ligand was not achievable. However, both ICI182,780 and GW7604 distinguished themselves from the other ER ligands examined in this assay as they permitted a robust and sustained suppression of ERα protein levels and this rapid loss of receptor could not be solely attributed to decreased ERα mRNA levels (Fig. 1C). Given these findings, we proceeded to determine whether or not these two compounds achieved the end point of receptor turnover in the same manner.

The SERDs ICI182,780 and GW7604 have differential effects on subcellular localization of ERα. It has been shown previously that although treatment with either estradiol or ICI182,780 results in a quantitative down-regulation of ERα in cells, the mechanism(s) by which this occurs is not the same (30–32). Most notable is the observation that these compounds have discrete effects on receptor compartmentalization, which precedes the degradation of the receptor (33–36). This suggests that different ligand-induced changes in the structure of ERα result in the receptor entering the proteolytic pathways through alternate mechanisms. We were interested in determining if the distinct cellular compartmentalization of ERα, observed following treatment with ICI182,780, was a hallmark of SERDs or if it was specific to this steroidal antagonist. To this end, we compared the kinetics of receptor distribution in MCF-7 cells following treatment with vehicle alone, estradiol, or with the SERDs ICI182,780 or GW7604. The treated cells were harvested and cellular extracts were fractioned into soluble cytoplasmic, soluble nuclear, soluble DNase/ammonium sulfate/high NaCl treated, and the remaining insoluble fraction (Fig. 2). In this manner, it was observed that ERα was found in either the cytoplasmic or insoluble fractions following treatment with vehicle (Fig. 2A–C). Treatment of cells with estradiol resulted in a higher recovery of ERα in the soluble nuclear and DNase/ammonium sulfate/high NaCl fractions at earlier time points with progressively more receptor associating with the insoluble fraction as the duration of exposure increased (Fig. 2A). In contrast, the ICI182,780-occupied ERα was found to associate predominantly with the insoluble fraction (Fig. 2B). Using the same approach, we observed that the localization pattern for receptors treated with GW7604 was quite similar to that observed for estradiol and surprisingly distinct from the ICI182,780-treated receptor (Fig. 2C). Lamin A (nuclear) and α-tubulin (cytoplasmic) were measured in all extracts to assess the effectiveness of the fractionation protocol and to verify that these ligands did not have a nonspecific effect on protein compartmentalization. Thus, although ICI182,780 and GW7604 are classified as SERDs, they do not have the same effect on the compartmentalization of ERα within the cell. Although it is not possible to infer from this data that ICI182,780 and GW7604-dependent ERα turnover is related in any way to cellular localization, it does indicate that the cell does not handle the receptor in the same manner when occupied by these different SERDs. We conclude from these studies that the rapid repartitioning of ICI182,780-treated ERα from a soluble to an insoluble fraction precedes degradation and is a unique characteristic of this subclass of SERDs.

ERα adopts a unique structural conformation in the presence of ICI182,780. It is now generally accepted that many of the functional differences exhibited by different ER ligands are related to (a) their ability to induce distinct alterations in receptor structure and (b) the ability of differently conformed receptors to engage different cofactors in target cells. We inferred, therefore, that if the SERDs ICI182,780 and GW7604 are indeed mechanistically distinct, as the data presented in Fig. 2 indicates, then the structure of the receptor in the presence of these molecules should be sufficiently different so as to enable the differential engagement of cofactors. This idea is supported by our previous observation that the degree of hydrophobicity exhibited by the ERα ligand-binding domain is not equivalent in the ICI182,780 and GW7604 complexes (23). Thus, we next examined whether or not these two mechanistically distinct SERDs induce different structural alterations in ERα. To address this issue, we used combinatorial T7 phage display to screen random peptide and cDNA expression libraries for peptides/proteins that interact in a specific manner with ICI182,780-activated ERα. This was accomplished using purified ERα immobilized in plastic to select for phages expressing either an interacting peptide or cofactor. Interestingly, although this approach led to the identification of a large number of peptides and proteins that interacted with estradiol-activated ERα (data not shown), we identified only a single peptide (AEIP) that interacted specifically with the ICI182,780/ERα complex. Further characterization of the receptor-interacting properties of this peptide was done using a mammalian two-hybrid assay (Fig. 3). For this assay, we expressed AEIP as a fusion protein with the GAL4-DBD and assessed its ability to interact with a full-length VP16-ERα fusion protein in the presence of different ligands. The results of this analysis indicate that this peptide recognizes a surface presented on the ICI182,780/ERα complex that is not present on the receptor when it is occupied by GW7604 (a SERD) or other SERMs (tamoxifen, raloxifene, and EM-652; Fig. 3A). Furthermore, we showed that the interaction of AEIP with ERα could be reversed by the addition of increasing concentrations of estradiol, confirming that ICI182,780 and estradiol-activated ERα are structurally distinct (Fig. 3B). Finally, GAL4-DBD-AEIP did not interact with any of the other nuclear receptors that were tested, including both the long (amino acids 1–530) and short (amino acids 1–477) VP16-ERβ isoforms (Fig. 3C). This was an extremely important result, as we and others have shown that ERβ expression is not influenced by ICI182,780 (37). These data provide compelling evidence that potentially, a specific surface on ERα, presented upon binding ICI182,780, results in distinct receptor re-compartmentalization and subsequent degradation. Thus, within the confines of the resolution of this assay, we conclude that the structure of ICI182,780-activated ERα is distinct from the nonsteroidal antiestrogens evaluated in this assay.

Sequence analysis of AEIP revealed that it was composed of a total of 42 amino acids (Fig. 3D). In order to define the ERα-interacting domain of this extended peptide, we sequentially mutated six amino acid regions across the entire sequence using a NAARIS replacement strategy (38). A schematic illustrating the regions mutated is shown in Fig. 3D. When the AEIP NAARIS mutants were used in M2H assays, it was observed that the amino acids within region 5 were responsible for the interaction with ERα (Fig. 3D). This region contained a high number of hydrophobic amino acids, which is interesting given that E2-bound ERα has a high affinity for the hydrophobic amino acid sequence LxxLL found in coactivator proteins (26, 39, 40). To further determine which of the six amino acids were required for the interaction, each amino acid was individually mutated to alanine (A) or in combination at positions 1 and 2 because we thought it was possible these hydrophobic amino acids might compensate for one another. M2H assays revealed that the last three amino acids (S28, P29, and M30) were key amino acids involved in the interaction (Fig. 3D). Thus,
AEIP contains a discrete hydrophobic domain that is required for interaction with ICI182,780-activated ERα.

**Conformation as a predictor of ERα stability.** Over the past few years, it has become apparent that there is a strong relationship between the structure of an ER-ligand complex and resultant biological activity (26, 27, 39–41). Thus, we felt that it was likely that any compound that facilitates the interaction of AEIP with ERα would induce its turnover. To test this hypothesis, we used a mammalian two-hybrid assay as a primary screen to identify from a library of ER ligands those that could facilitate the interaction of ERα with AEIP. Only a single compound, the pure antiestrogen RU58,668, facilitated a significant interaction of ERα with AEIP (Supplemental Fig. S1A; ref. 42). As expected, RU58,668 induced a rapid and sustained decrease in ERα protein levels that could not be attributed to a decrease in ERα mRNA levels alone (Supplemental Fig. S1B and C). These
findings provide a strong link between receptor conformation and stability and provide the validation for a mechanism-based screen with which to identify SERDs with improved pharmacological properties.

**Definition of the functional significance of the AEIP binding surface on ERα.** It is our assumption that the surfaces presented on ERαs in the presence of IC1182,780 allow its interaction with specific proteins that target it for degradation. As of yet, we have been unable to identify proteins that are capable of interacting with this specific receptor-ligand complex, and thus, are unable to test this hypothesis directly. Furthermore, because the crystal structure of ERα in the presence of IC1182,780 has not yet been solved, we have had to rely on mutagenesis to determine what region and amino acids of ERα constitute the AEIP interaction surface(s). This is an important next step in understanding the mechanism of action of this class of SERDs as it will hopefully lead to the definition of the mechanistic link between ERα conformation and stability. The goal in the next series of studies, therefore, was to characterize the AEIP interaction domain on ERα and determine if this region is directly involved in the regulation of receptor stability or if its presentation is merely a surrogate for more functionally important structural alterations that occur at another surface on the protein.

In the first series of studies, we compared the effect of a series of receptor mutations, created in the background of VP16-ERα, on the receptor-binding activities of AEIP (Fig. 4A). These specific mutations were selected as they have previously been shown to alter the interaction of ERα with coactivators and corepressors (26, 27, 39, 43). For comparative purposes, we also examined the interaction of these mutants with (a) the NR-box of SRC-1 and (b) a peptide that interacts with tamoxifen-activated ERα (Fig. 4B and C; ref. 27). As observed in Fig. 4A, the interaction of AEIP with IC1182,780-bound ERα was abrogated when the amino acid I358 within helix 3, L379 in helix 5, and the hydrophobic amino acids of helix 12 were mutated. Interestingly, with the exception of amino acid K362, which mediates a capping interaction with LxxLL-containing coactivators (i.e., SRC1), the mutations shown to abrogate the interaction of AEIP with IC1182,780-bound ERα were the same as those necessary for E2-induced interaction of the SRC1 NRID (peptide region of SRC1 containing all three LxxLL; Fig. 4A and B; ref. 39). These mutant receptors were capable of interacting with a peptide that binds to ERα in a ligand-independent manner, indicating that the selected amino acid changes did not induce a global alteration in receptor structure (data not shown). We have previously identified a peptide (bT1) whose binding surface is presented only on tamoxifen-activated ERα (27). Analysis of the effects of the selected mutants on the interaction of this peptide with ERα has led us to conclude that the surfaces presented on the IC1182,780-activated ERα are dramatically different from those presented upon tamoxifen binding (Fig. 4C). Finally, Kong et al. have used crystallography to identify a novel protein-protein interaction domain that is presented on ERα following its interaction with any ligand (43). The functionality of this domain is disrupted by the G424H mutation. However, this mutation has no effect on the interaction of the receptor with AEIP. Thus, we conclude from these mutagenesis experiments that the conformation of ERα adopted in the presence of estradiol and IC1182,780 are unexpectedly quite similar. Furthermore, we consider it likely that the AEIP and coactivator binding surfaces overlap.

**The integrity of the AEIP binding site is required for IC1182,780-mediated subcellular trafficking of ERα.** The earliest response to IC1182,780 we have observed is the re-partitioning of ERα from a soluble to an insoluble fraction; an activity that tracks with degradation (Fig. 2B). Thus, in order to test the functional role of the AEIP binding surface directly, we examined the cellular partitioning of each mutant in the presence of IC1182,780 (Fig. 5). To avoid the confounding effect of endogenous ERα that may dimerize with some of the mutants, we did these studies in transiently transfected ERα (–) HeLa cells. For these studies, a V5 tag was added to the NH2 terminus of each protein to allow quantitation by Western immunoblot. We first confirmed that we could observe the IC1182,780-induced subcellular compartmentalization of exogenously expressed ERα in HeLa cells, as was observed with the endogenous receptor in MCF-7 cells (Fig. 5A). In these studies, because ERα was never observed in the soluble nuclear or DNase fractions following IC1182,780 treatment (Fig. 2B), we only assessed its partitioning between the low salt–soluble and low salt–insoluble fractions. As observed previously, we did not see a decrease in total ERα levels following 90 min of treatment, allowing us to examine the early compartmentalization events (Fig. 5A; ref. 36). However, we did observe a rapid loss of ERα in the low salt–soluble fractions and a concomitant increase in insoluble receptor following 15 min of treatment (Fig. 5A). Using this system, we proceeded to evaluate the effect of mutating the AEIP binding site on ERα compartmentalization. To this end, we expressed ERα, ERβ, or each ERα mutant individually in HeLa cells and assessed their compartmentalization

Figure 3. Identification of a peptide that interacts specifically with the IC1182,780-bound ERα. A, mammalian two-hybrid assays were done in HepG2 cells as described in Materials and Methods by transfecting a 5XGal4-TATA-luc reporter, a VP16-ERα expression plasmid, and either GAL4-DBD alone or GAL4-DBD-AEIP expression plasmid. Cells were treated with vehicle or 100 nM/L of the following ligands: E2, TOT, GW7604, raloxifene, EM-652, or ICI182,780, for 24 to 36 h. Transcriptional activation of 5XGal4-TATA-luc, and therefore ERα/AEIP interaction, was measured with a luciferase assay and activity was normalized for transfection efficiency with transfected CMV-β-gal plasmid. Columns, average of at least three triplicate experiments for all ligands except raloxifene (two triplicates); bars, SE. B, mammalian two-hybrid assays were done as previously described and cells were treated with vehicle, IC1182,780 alone, or with IC1182,780 in combination with E2 at various concentrations from 0.1 to 100 nM/L. ERα/AEIP interaction was measured as in (A). Columns, averages of two triplicate experiments; bars, SEs. C, mammalian two-hybrid assays were done as described using GAL4-DBD-AEIP and VP16-ERαs, -ERβ long form (L), -ERα short form (S), -androgen receptor (AR), -glucocorticoid receptor (GR), -progesterone receptor A or B form (PRA or P RB), or retinoic acid receptor (RAR). Cells were treated with either vehicle (V), 100 nM/L of IC1182,780 (IC1), E2, casodex (Cas), R1881, RU486 (486), dexamethasone (Dex), RU5020 (2020), or 9-cis retinoic acid (9-cis) depending on the transfected receptor. GAL4-DBD-AEIP interaction with various receptors was determined by luciferase expression as in (A), and compared with GAL4-DBD interaction alone. Columns, average of ERα, ERβ, L, AR, and PRA interaction with AEIP from two triplicate experiments and the average of ERα, S, GR, PRB, and RAR interaction with AEIP from one triplicate experiment; bars, SE. D, diagram of AEIP amino acid sequence and amino acid sequences of mutated peptides. WT AEIP, amino acids of AEIP beyond the GAL4-DBD; NAAIRS mutants, the seven regions (1, 2, 3, 4, 5, 6, 7) of the AEIP peptide that were individually mutated to the NAAIRS amino acids and acid sequence in the context of the wild-type peptide. Alanine mutants, the amino acids that were mutated to alanine either individually (1, 2, 3, 4, 5, 6, or 7) or together (1 & 2). The specific amino acids in AEIP necessary for the pure antiestrogen-induced AEIP/ERα interaction (underlined). Mammalian two-hybrid assays were done in HepG2 cells as previously described in Materials and Methods to determine the interaction of VP16-ERαs with various AEIP mutants. Bottom left, AEIP-NAAIRS mutants in the presence of vehicle or 100 nM/L of IC1182,780. Columns, the average interactions from three triplicate experiments; bars, SEs. Bottom right, AEIP alanine mutants in the presence of vehicle or 100 nM/L of IC1182,780. Columns, the average interactions from three triplicate experiments; bars, SEs.
in the presence of ICI182,780 as a function of time. As can been seen in Fig. 5B, those mutants that interacted with AEIP were all found to rapidly associate with the insoluble fraction. In contrast, those mutants that did not interact with AEIP failed to recompartmentalize under the conditions of our assay. The data presented in Fig. 5C and D represent the average level of soluble and insoluble ERα, ERα mutants, and ERβ following ICI182,780 treatment from three independent experiments. We believe that the robust nature of this data highlights the importance of the AEIP-interacting domain in regulating ERα trafficking, and likely, its stability.

Expression of the AEIP peptide in cells partially inhibits ICI182,780-mediated recompartmentalization of ERα. The data presented thus far support the hypothesis that the AEIP binding site on ERα is a protein-protein interaction surface required for ICI182,780-mediated subcellular translocation. Definitive proof of this hypothesis will require the identification of the protein(s) that use this surface; an ongoing project in our laboratory. In the interim, however, we have tested this idea by expressing AEIP in cells and observing the effect of this manipulation on the fate of ICI182,780-occupied ERα in target cells. Specifically, we constructed adenoviruses expressing either a GAL4-DBD-AEIP fusion protein or a GAL4-DBD-control (AEIP interaction domain mutant). We first confirmed that expressed GAL4-DBD-AEIP, but not the mutant protein, interacted with ERα using a M2H assay. For this assay, increasing amounts of viruses expressing each protein were used to infect HeLa cells and their ability to interact with a transiently transfected VP16-ERα was assessed using a GAL4-RE-Luc reporter. Equivalent levels of both proteins were expressed in cells. However, only the AEIP protein was found to interact with ERα in an ICI182,780-dependent manner (Fig. 6A).

We have validated the use of the recompartmentalization assay to study the fate of ICI182,780-activated ERα, and thus, again used this approach to assess the effect of AEIP expression on the partitioning of endogenous ERα. To this end, we infected MCF-7 cells with viruses expressing either AEIP or the mutant and measured ERα levels in the soluble and insoluble fractions of the cell following treatment with different ligands. The data obtained following 1 h of ICI182,780, shown in Fig. 6B and C, indicate that
AEIP, but not the mutant, increases the amount of ERα in the soluble fraction and decreases its recovery from the insoluble fraction. The levels of ERα in either compartment was unaffected by AEIP expression following treatment with ligands other than ICI182,780. Each assay was done thrice with similar results, confirming the ability of AEIP to block the movement of ICI182,780-treated ERα to the insoluble fraction. We conclude from these experiments that AEIP recognizes a specific surface on Figure 5. Specific amino acids responsible for the ICI182,780-bound ERα interaction with AEIP are also necessary for ICI182,780-mediated subcellular compartmentalization.

A, HeLa cells were transfected with CMV-β-gal, pEGFP, and pCDNA-V5-tagged ERα, treated with vehicle or 100 nmol/L of ICI182,780 for the specified time period, and lysed to obtain low salt-soluble and -insoluble fractions as previously described in Materials and Methods. The two fractions were subjected to Western analysis to detect V5-tagged ERα, and EGFP. Loading was initially controlled based on β-gal expression followed by EGFP expression.

B, HeLa cells were transfected with pEGFP, CMV-β-gal, and either pCDNA-V5-ERα [wild-type (WT) or mutants] or -V5-ERβ expression plasmids, treated for 3 h with either vehicle (V) or 100 nmol/L of ICI182,780 (ICI), harvested and lysed as in (A). The soluble and insoluble fractions were subjected to Western analysis to detect V5-tagged ERα WT, mutants, or ERβ, and EGFP. Loading was controlled as in (A). Western blots are representative of three independent experiments for each receptor. Relative soluble (C) and insoluble (D) ER levels following treatment versus vehicle as determined by densitometry readings using Image J and comparing ER density to EGFP density. Columns, average from three independent experiments for each receptor; bars, SEs.

Figure 5. Specific amino acids responsible for the ICI182,780-bound ERα interaction with AEIP are also necessary for ICI182,780-mediated subcellular compartmentalization.
ERα that is presented upon its interaction with ICI182,780, which enables a protein-protein interaction required for receptor compartmentalization, and likely, its degradation. We also conclude that although GW7604 is a SERD and resembles ERα in its ability to down-regulate ERα, it does so by a different mechanism as it is unaffected by the expression of AEIP.

**Discussion**

The utility of the antiestrogen tamoxifen in the treatment and prevention of breast cancer is well established (13). However, as with most first-in-class drugs, the insights provided from years of clinical exposure has revealed activities of this drug that may limit its clinical utility. With respect to tamoxifen, the most notable
deficiencies are (a) de novo resistance in some ERα-positive tumors, (b) acquired resistance with continued administration of the drug, (c) partial agonist activity in the uterus, and (d) increased risk of thromboembolic events (13, 21, 22). Thus, there is a high level of interest in developing novel antiestrogens which will display improved therapeutic activity and which will be used either in place of, or subsequent to, tamoxifen (22). Unfortunately, despite promising results in preclinical models, there has been relatively little success in identifying antiestrogens that either show superior activity to tamoxifen or which can inhibit tamoxifen-resistant breast tumors. For instance, whereas the SERM raloxifene, a molecule that is both chemically and functionally distinct from tamoxifen, exhibited a favorable profile in preclinical studies, it was shown to be ineffective as a treatment for tamoxifen refractory metastatic breast cancer in the clinic (44). Indeed, these and other disappointing clinical trials led to the widely held view that ERα was not a useful therapeutic target in patients whose tumors progressed on tamoxifen. However, this opinion was changed by the dramatic results observed in a phase II clinical trial ofICI182,780, a SERD (14, 16–20). The efficacy of this particular drug has been attributed to its ability to both inhibit ER transcriptional activity and its ability to induce receptor turnover. Given that ER can be activated in a ligand-independent manner by signaling pathways that modulate the receptor, or its associated cofactors, it is likely that the ability of SERDs to induce receptor turnover is a key contributor to its clinical efficacy (14, 16, 24, 30). Faslodex (ICI182,780) is now approved for use in the treatment of metastatic disease, and although it is clearly a breakthrough medicine, its clinical utility is limited by its poor bioavailability and the length of time that it takes to achieve a useful therapeutic concentration in target tissues (14, 18, 20, 22). It was within the framework of this clinical problem that we embarked several years ago on the identification of new molecules that exhibit SERD activity. This work led to the identification of GW5638, an orally active, nonsteroidal tamoxifen derivative, which, like ICI182,780, facilitates a rapid turnover of ERα and effectively inhibits the growth of tamoxifen-resistant tumors propagated in athymic nude mice (22, 45). The current study extends this initial work and shows that although both ICI182,780 and GW5638, and its bioavailable metabolite GW7604 are members of the SERD subclass of ER ligands, they effect a down-regulation of the receptor by different mechanisms. This is an important finding as it suggests the possibility that these drugs, or molecules with similar mechanism(s) of action, could be used sequentially and/or they may be useful in patients whose tumors exhibit different molecular characteristics. Furthermore, given the limitations of ICI182,780, we considered that by understanding its mechanism of action, we may be able to develop predictive screens that would enable the identification of molecules with similar activities but show improved pharmacuetical properties.

It is now well established that the pharmacologic activities of different ER ligands are determined by the effects of each ligand on receptor structure and the secondary effect that this has on cofactor recruitment (26, 27, 39–41). We have previously shown, using a variety of biochemical and biophysical techniques, that the structures of ERα in the presence of ICI182,780 or GW5638 (GW7604) were not identical and suspected that this may translate into important mechanistic differences, secondary to differential cofactor recruitment (23, 24, 30). The identification of AEIP, a peptide that interacted specifically with ICI182,780-bound ERα supports this link between a specific molecular structure and receptor degradation. We have also observed that RU58,668, a steroidal pure antiestrogen, also facilitates ERα-AEIP interaction and induces receptor turnover. This important information provides compelling support for the link between a specific ERα conformation and stability. Importantly, AEIP did not bind to GW7604-activated ERα. However, using combinatorial peptide phage display, we have shown in the past that the surfaces presented on GW7604 or GW5638-activated ERα are generally not present when the receptor is occupied by ICI182,780 (24). We conclude therefore that ICI182,780 and GW5638 (GW7604) are members of two distinct subclasses of SERDs.

Mutational analysis of AEIP identified a hydrophobic region within the peptide that interacts with ICI182,780-bound ERα. This was reminiscent of the hydrophobic regions of cofactors which are known to be involved in binding to both agonist- and SERM-bound ERα (26, 27, 39, 40). This led us to further examine the region of ERα with which AEIP was binding. It was observed that AEIP interacts with a similar, but slightly different, hydrophobic pocket than that used by estrogen-bound ERα for coactivator binding and was completely different from that used for interaction with CoRNR box-like peptides (27, 39). This suggested that the ICI182,780-induced conformation is more similar to that induced by estrogen than SERMs. Furthermore, based on these studies, we suspect that the extended sulfoxide-containing alkyd side chain of ICI182,780 is pushing helix 12 into the coactivator binding pocket, blocking coactivator binding but allowing for the AEIP peptide to bind. The dependence on helix 12 within ERα for AEIP interaction indicates why this peptide was unable to bind ICI182,780-bound ERβ. Crystal structures of ICI182,780-bound ERβ have been unable to resolve helix 12, suggesting that this region of ERβ is highly mobile and unstructured when bound to ICI182,780 (46), and thus, it is likely that the conformation induced by steroidal SERDs within ERα is different from that induced in ERβ. Studies are under way to crystallize ICI182,780-bound ERα in the presence of AEIP, which should help resolve this issue.

Our studies and those published by others have shown that following ICI182,780 treatment, ERα is rapidly translocated to a highly insoluble, nuclear matrix–associated fraction of the cell, whereupon it undergoes degradation (34–36). The recent demonstration that the solubility of ERα bound to ICI182,780 is influenced by the integrity of helix 12 supports the conclusions of our studies (36, 47). However, we have shown that additional mutations within the ligand-binding domain of ERα that disrupt AEIP-binding alter receptor compartmentalization and increase its stability. Specifically, we have shown that helices 3 and 5 are also involved in ERα/ICI182,780 complex and the binding of AEIP. In addition, these findings support the existence of an endogenous protein, containing a motif similar to that observed in AEIP, that regulates the trafficking and stability of the receptor. This hypothesis was further supported by the observation that expression of AEIP in cells was able to partially block ICI182,780-mediated turnover of ERα.

Computational analysis using the National Center for Biotechnology Information BLAST program resulted in the identification of only one protein (ZMYM4) with an exact match to the AEIP motif (VPNSPM). In addition, a large number of many unknown proteins, and some known to be potentially involved in cellular trafficking such as trafficking kinesin-binding protein 1, were identified as containing the PNSPM sequence. It will be of interest to determine if any of these proteins are involved in determining the molecular pharmacology of ICI182,780. Regardless, the identification of AEIP and the demonstration that its binding determined the fate of ERα has, at a minimum, provided a facile assay for the identification of novel ICI,182,780-like SERDs.
In summary, we have determined that the currently available SERDs can be divided into two mechanistically distinct classes based on their differential effects on receptor structure and in the manner by which these differently configured receptors are recognized within cells. This important finding predicts that, notwithstanding non-mechanism-associated activities, the currently available steroidal antiestrogens will exhibit cross-resistance in breast tumors (48), but that the benzothiophene-derived SERD, GW5638, is sufficiently unique as to expect that it can be used in patients that are resistant to molecules like ICI182,780, as has been previously shown in tumors resistant to tamoxifen (24, 45).

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Definition of Functionally Important Mechanistic Differences among Selective Estrogen Receptor Down-regulators

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