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The molecular mechanisms underlying the pharmacological actions of ER modulators: Implications for new drug discovery in breast cancer

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Introduction

Our understanding of the molecular mechanisms underlying the pharmacological actions of estrogen receptor (ER) ligands has evolved considerably in recent years. Much of this knowledge has come from a detailed dissection of the mechanism(s) of action of the Selective Estrogen Receptor Modulators (SERMs) tamoxifen and raloxifene, so called for their ability to function as ER agonists or antagonists depending on the tissue in which they operate. These mechanistic insights have had a significant impact on the discovery of second generation SERMs, some of which are in late stage clinical development for the treatment/prevention of breast cancer as well as other estrogenopathies. In addition to the SERMs, however, have emerged the Selective Estrogen Degraders (SERDs), which as their name suggests, interact with and facilitate ER turnover in cells. One drug of this class, fulvestrant, has been approved as a third line treatment for ER-positive metastatic breast cancer. Whereas the first generation SERMs/SERDs were discovered in a serendipitous manner, this review will highlight how our understanding of the molecular pharmacology of ER ligands has been utilized in the development of the next generation of SERMs/SERDs, some of which are likely to have a major impact on the pharmacotherapy of breast cancer.

The evolution in our understanding of ER pharmacology

Drugs like tamoxifen and raloxifene were initially classified as antagonists and were developed as agents that could competitively displace estradiol from ER and inhibit its mitogenic actions in breast cancer cells [1,2]. However, it was apparent even from the earliest studies of these drugs in animals that their pharmacology was substantially more complex and that they were capable of exhibiting agonist, partial agonist or antagonist activities in different tissues [3–6]. Regardless, it was not until much later that it became clear that tamoxifen and raloxifene were more appropriately classified as Selective Estrogen Receptor Modulators (SERMs) [7]. One of the most important paradigm shifting experiments was that performed by Gottardis and Jordan in the late 1980s when they showed, in xenograft models of breast cancer, that whereas

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tamoxifen initially functioned as an ER antagonist, over time the tumors developed resistance to the drug and eventually switched to recognizing it as an agonist [8]. These data indicated that the pharmacology of tamoxifen was not stable and that over the course of chronic treatment something changes within the target cell that enables it to recognize the ER-tamoxifen complex as transcriptionally "active". There were also anecdotal reports in the clinical literature of withdrawal responses in patients who progressed while on tamoxifen, a result that supported the observation that even in breast cancer cells tamoxifen could exhibit both antagonist and agonist activities [9]. It was also of significance that clinical studies revealed that both raloxifene and tamoxifen exhibited robust ER agonist activities in bone, and that tamoxifen and structurally related molecules manifested estrogenic activity in the uterus [10,11]. Taken together these data indicated that (a) a given compound, acting through the same receptor, can manifest different activities in different cells, (b) subtle differences in the structure of ER ligands can result in significantly different phenotypic responses, and (c) alterations in the cellular environment in which ER operates can dramatically alter the pharmacology of its bound ligands. The importance of these observations in understanding the pharmacological actions of existing ligands as well as in developing approaches for new ligand discovery has driven efforts directed at defining the cellular mechanisms that enable cells to distinguish between different ER-ligand complexes.

The molecular mechanisms underlying the pharmacological actions of ER ligands

In the absence of ligand ER associates with a large heat shock protein complex in either the nucleus or cytoplasm of target cells. Upon binding an agonist, the receptor undergoes a conformational change that initiates a cascade of events that enables its interaction with the regulatory regions of target genes. Interestingly, although ER has a well defined DNA binding domain and can interact with specific estrogen response elements (EREs) within target genes, it also uses the same domain to interact with DNA in an indirect manner by tethering to proteins such as AP1 or Runx1 that are bound to their cognate response elements [12,13].One of the major changes in our understanding of ER action, however, is that we no longer consider the receptor itself as directly impacting the activity of the transcriptional apparatus. Rather, it serves as a nucleating point for transcriptional coregulators, proteins with different enzymatic activities that can directly modify chromatin structure and/or impact the activity of the general transcription apparatus. Thus, it is the activity of the coregulators recruited by ER, rather than the receptor itself, that is the determinant of the response of the target gene to a specific receptor-ligand complex. There are over 300 proteins that have been shown to interact with one or more members of the nuclear receptor superfamily, a significant number of which can interact with ER.Not surprisingly, there has been a tremendous amount of work performed at a genetic and biochemical level to study the function and mechanism of action of these coregulators in ER pharmacology/physiology. Emerging from these studies are important "tenets" that describe how information is transferred from different ER ligand complexes to the transcription apparatus. Specifically it has been demonstrated that (1) the overall shape of ER is determined by the nature of the ligand with which it is bound, (2) receptor conformation regulates the differential interaction of the receptor with functionally distinct transcriptional coregulators, and (3) the relative and absolute levels of transcriptional coregulators differ between cells [14]. Furthermore, it is now clear that post-translational modifications of the receptors and/or the coregulators with which they interact can also impact the activity of the receptor-ligand complex at target genes. Additional complexity is introduced when one considers that there exist two genetically distinct estrogen receptors, ERα and ERβ that have independent activities when acting as homodimers or can modulate each other's activity through heterodimerization in cells where they are both expressed [15,16]. While the ERα and ERβ DNA binding domains share high homology, it has been demonstrated by several investigators that in addition to being

able to regulate the expression of similar target genes, each receptor sub-type also exhibits distinct target gene repertoires [17,18]. The molecular basis for this discrimination is not known, but undoubtedly it will be found to involve the differential actions of attendant cofactors. Interestingly, the two ER subtypes also exhibit significant differences in their response to pharmacological agents [19]. Notable was the observation that whereas tamoxifen inhibits transactivation by ERα, it can function as an ERβ agonist when analyzed on the same target gene under the same conditions [18]. Furthermore, while the SERD ICI 182,780 interacts with both ERα and ERβ with high affinity, it only induces turnover of ERα within cells [20]. It is worth noting that the expression of ERβ in ERα-positive breast cancer cells and xenograft tumors decreases ERα transcriptional activity and reduces the proliferative response to estrogens. However, the expression of ERβ in ERα-negative models of breast cancer increases cell proliferation [21,22]. Clearly, the important differences in ERα and ERβ pharmacology need to be considered in the design of the next generation of ER-modulators for the treatment and prevention of breast cancer.

Another important advance in our understanding of ER action comes from a reassessment of the definition of a “ligand”, historically considered a small lipophilic molecule that interacts with the ligand-binding domain of the receptor. It is now appropriate to consider that in addition to steroidal agonists, the DNA sequence and the coregulators with which the receptor interacts can impact its overall structure and modulate its biochemical properties [23**–25]. The relevance of these alternate modes of activation was reinforced in studies where it was shown that overexpression of a positive coregulator alone was sufficient to enable ER transcriptional activity. Furthermore, it was demonstrated that the binding kinetics of ER and estradiol were influenced by coregulator induced allosteric changes in the structure of the receptor ligand binding domain [23**]. Cumulatively these findings suggest that in addition to the classical ligand-binding pocket, it may be possible to target additional regulatory surfaces on ER in the search for new classes of receptor modulators.

Given our current understanding of the factors/processes that impact ER function, it is now clear how subtle differences in the structure of a ligand can have profound effects on its pharmacological activity. Furthermore, given the relationship between receptor conformation and activity, it is not surprising that molecular screens capable of detecting specific conformational states of ERα were used in the identification of lasofoxifene and bazedoxifene, second generation SERMs that exhibit distinct biological activities [7,26,27]. This and other information highlight the importance of receptor conformation in determining the pharmacological activity of different compounds and provide a mechanism to achieve functional diversity in ligands. On the other hand, the complexity of these pathways has also highlighted why it has been and will continue to be difficult to develop ER ligands whose pharmacological properties remain stable over time and which can be used for the chronic treatment of breast cancer.

The impact of clinical research on our understanding of ER pharmacology

The majority of breast tumors express ERα and thus tamoxifen (or related SERMs) or aromatase inhibitors have become frontline therapeutic interventions in this disease. Consequently, there is a tremendous amount of clinical information on the performance of ER signaling modulators in breast cancer. Consideration of this data, together with what is known about the mechanism of action of these agents, is instructive with respect to pharmacological characteristics required of the next generation of therapeutics that target this receptor. Whereas it is also likely that ERβ has a role to play in opposing ERα action in the breast, it is likely that it is the targeting of ERα that provides the majority of the therapeutic benefit [28].

When used in the metastatic setting tamoxifen effectively halts breast tumor progression with a duration of response in the range of 18–24 months.As described above, it is believed that treatment failure represents a switch in the environment of ERα that enables the cell to recognize tamoxifen as an agonist. This hypothesis is supported by data from studies of tamoxifen use in the adjuvant setting, which indicated that patients treated for 5 years with tamoxifen did significantly better than patients treated for longer periods (up to 10 years) [29]. Thus, it appears that rather than simply having no added benefit there was something about longer duration of exposure to tamoxifen that actually caused it to do harm.Given what is now known about ER signaling and coregulator biology, it has been proposed that chronic exposure to tamoxifen, either in the metastatic or adjuvant setting, induces resistance by (a) selecting for a population of cells within the tumor that has in place proteins and processes that enable this drug to manifest agonist activity, or (b) inducing an epigenetic change that results in the expression of components of the transcription apparatus that permits tamoxifen to function as an agonist.Regardless of which of these very similar mechanisms is correct, it is inferred that since ERα is a transcription factor, resistance represents a gain of function activity that results from the ability of the tamoxifen ER-complex to interact with a transcriptional coregulator(s) that enables it to manifest agonist activity. Thus, differences in the expression and/or activity of coregulators are now considered to be primary determinants of tamoxifen agonist/antagonist activity.

There is a substantial amount of experimental evidence that points to the primacy of coregulators in determining ER-ligand pharmacology. One particularly important finding came from the work of Smith et al which demonstrated that overexpression of the transcriptional coregulator SRC-1 alone was sufficient to confer upon cells the ability to recognize tamoxifen as an agonist [30*]. This suggested that although tamoxifen induces a conformational change in ERα that dramatically reduces its ability to interact with coactivators, the impact of this disruptive conformational change can be overcome by increasing the cellular concentration of a specific coactivator. This, coupled with the fact that tamoxifen enables efficient delivery of the receptor to DNA and that it also significantly reduces ERα turnover, explains how this drug can induce significant activation of ER target gene transcription [31]. It is noteworthy, in this regard, that it has been shown that elevated expression of SRC-1 and/or SRC-3 is associated with tamoxifen resistance and that the locus encoding SRC-3 is amplified in a large number of breast cancers [32–34*].

Growth factor receptor signaling modulates ER activity and ligand pharmacology

There is a considerable amount of data to indicate that the development of resistance to tamoxifen is associated with an increase in Her2 expression in breast cancer cells [35]. This compensatory response has been shown to occur in both cellular and animal models of this disease.It has also been shown that treatment of Her2-overexpressing, tamoxifen-resistant breast cancer cells with either the Her2 antagonist trastuzumab or with the dual Her2/EGFR tyrosine kinase inhibitor Lapatinib, is sufficient to restore tamoxifen sensitivity [36]. Furthermore, IGF-1R/EGFR mediated activation of PKA or MAPK signaling pathways *in vitro* has been shown to result in ligand-independent activation of ERα transcriptional activity and also to increase the efficacy/potency of agonists and partial agonists like tamoxifen [37, 38]. Thus, aberrant activation of several different signaling pathways can impact ER pharmacology. One possible explanation for these responses is that Her2/IGF1R activation results in increased phosphorylation of sites on ERα that are associated with ligand activation and that this facilitates coregulator recruitment. Indeed, Her2 and IGF1R expression and phosphorylation, as well as phosphorylation of ER, were found to be prominent in tumors exhibiting *de novo* or acquired resistance to tamoxifen [39]. However, although there are several studies that highlight a very good correlation between increased phosphorylation and

ERα transcriptional activity, a definitive cause and effect relationship remains to be established. Indeed, it has been shown recently that the dramatic effects of PKA on ERα transcriptional activity are likely due to phosphorylation of the coregulator CARM1 rather than direct receptor phosphorylation [40]. Furthermore, a careful analysis of the stoichiometry of phosphorylation following MAPK activation failed to demonstrate a significant increase in receptor phosphorylation, but rather revealed that phosphorylation of its attendant cofactor, SRC-3, was significantly increased [41]. Thus, it appears that activation of Her2 (or IGF1R) results in increased SRC-3 activity and subsequent increases in the agonist efficacy of estradiol and tamoxifen. It is interesting to note that patients with ER-positive tumors that express elevated Her2 and SRC-3 have the poorest overall survival and are least likely to respond to tamoxifen [33].

Whereas a functional relationship between increased expression and/or activity of SRC-3, activation of Her2 and alterations in ERα pharmacology has been appreciated for some time, it was not until recently that the biochemical basis for these interactions became apparent. Notable in this regard was the observation that resistance to the dual Her2/EGFR antagonist (Lapatinib) was associated with increased expression of ERα and that sensitivity to this inhibitor could be restored by treating the cells with the pure antiestrogen Fulvestrant [42*]. Equally important was the identification of an ERE within the regulatory region of the Her2 gene, through which the estradiol or tamoxifen occupied ERα repressed transcription of the Her2 gene by recruiting the transcriptional repressor PAX2 [43**]. However, it was observed that when SRC-3 expression and/or activity increased in cells, it was able to out compete PAX2 for ERα binding and repression of Her2 transcription was relieved. Interestingly, it has been shown recently that increased SRC-3 expression is an early response to tamoxifen administration [44]. Together these findings highlight the central role of SRC-3 in ERα signaling in breast tumors and demonstrate how differences in the expression and/or activity of coregulators can dramatically alter the pharmacology of ER ligands. A model describing what we know about ER/Her2 crosstalk and its ability to influence ER pharmacology is detailed in Figure 1.

Transcriptional coregulators as determinants of the pharmacological activity of ER ligands in breast cancer

Whereas the discussion above has focused on SRC-3, it is clear that there are many additional coregulators, the dysregulated expression and/or activity of which are likely to impact endocrine treatment of breast tumors. For instance, it has been shown that the NR coregulator HoxB13 is overexpressed in tamoxifen-resistant breast cancers [45,46]. Interestingly, diagnostic tests that measure HoxB13 expression are routinely used to predict the likelihood of response to tamoxifen [47]. However, the mechanism(s) by which this coregulator impacts ER signaling and pharmacology in breast cancer remains unclear. Another interesting coregulator is RTA-1/Fox2, a regulator of RNA splicing whose expression level alters tamoxifen pharmacology [48]. Interestingly, many known coregulators are among the known targets of this splicing regulator, although the precise mechanism by which differential splicing impacts ERα pharmacology remains to be determined [49].

It should be apparent from this discussion that it is differences in coregulator activity and expression, rather than biochemical changes in ERα itself, that determine the activity of receptor bound ligands. It is not surprising, therefore, that there is a considerable amount of interest in targeting the ERα-coregulator interface directly, using molecules that bind to the coregulator binding pockets on the receptor, or indirectly, using molecules that inhibit the activity and/or expression of coregulators [50,51]. Although protein-protein interaction surfaces are generally large and difficult to target with small molecules, the results of efforts in this direction suggest that this general approach may be feasible.

Exploitation of the mechanistic complexity in ER signaling for new drug development

Two recent findings from studies of the mechanism of action of ER that bear significance with respect to new drug discovery are (a) ligands induce different conformational changes in receptor structure which then engender different cofactor interactions and (b) there are mechanisms other than occupancy of the receptor ligand binding pocket by a small molecule agonist that can result in ER transcriptional activation. Considerable progress has been made in exploiting these findings in the development of new ER modulators. Two specific examples of the progress made in this regard will serve to illustrate this point.

(a) Development of molecules that engender different conformational changes in ER structure

One of the first attempts to develop molecules that inhibited tamoxifen-resistant breast cancer led to the identification of keoxifene (now called raloxifene), a high affinity ER ligand that was structurally unrelated to tamoxifen. The primary rationale at the time for this approach was that resistance to tamoxifen was thought to occur either as a consequence of ERα mutations that disrupted tamoxifen binding or because the drug itself was modified in such a way to alter its pharmacological properties. However, keoxifene (raloxifene) was not found to be effective in breast cancer and its development was discontinued [52]. In addition to unfavorable pharmaceutical properties, we now know from an abundance of structural studies that the overall conformation of the ERα-tamoxifen and ERα-raloxifene complexes are extremely similar and it is likely that they would interact with the same cofactors [53–55]. Therefore, the cross-resistance observed in the clinic was not surprising, and neither was their similar efficacy in breast cancer prevention as noted in the Study of Tamoxifen and Raloxifene (STAR) trial [56–58]. It was inferred, however, from the studies of tamoxifen/raloxifene pharmacology that compounds that enabled ERα to adopt a distinctly different conformation and that disrupted specific receptor cofactor interactions may have utility in the treatment of tamoxifen refractory tumors. To test this hypothesis, we developed a series of *in vitro* screens that facilitated the identification of compounds that enabled ER to adopt a conformational state distinct from those induced by tamoxifen, raloxifene or estradiol. In this manner, GW5638/DPC974 was identified, a compound that was subsequently shown to interfere with ERα action by directly disrupting the folding of the critical helix 12 in the ligand binding domain of the receptor [59*,60]. Importantly, this molecule had excellent pharmaceutical properties and inhibited the growth of both tamoxifen sensitive and resistant tumor xenografts. Most notably, in a small investigator-initiated clinical trial of the drug there was evidence of efficacy in patients with heavily treated metastatic disease. Unfortunately, a victim of corporate mergers and portfolio reviews, this drug was not determined to be a financial winner and was discontinued. Regardless, the work with this drug firmly established the concept that it was possible to manipulate ERα structure and identify compounds that could be used in the pharmacotherapy of tamoxifen-resistant ER-positive tumors. It will be of interest to see whether compounds like bazedoxifene that induce unique structural changes in ERα will be effective in the treatment of tamoxifen refractory breast cancers.

(b) Development of selective estrogen receptor degraders

Although it is possible to manipulate ERα structure and regulate its cofactor interaction preferences, it should be apparent from the information presented above that the mere presence of the receptor itself makes possible the engagement of a cofactor that will enable ER to manifest transcriptional activity. As proposed for tamoxifen resistance, selective pressure could result in the selection of a population of cells within a tumor that express a suitable cofactor and/or an existing cofactor made more compatible with the ER-tamoxifen complex

subsequent to the activation of a signaling pathway. These observations suggested that molecules resulting in ERα destruction may be particularly useful in the treatment of breast cancer (Figure 2). Indeed, this approach was fuelled in part by the observation that high-dose estrogens, which led to a rapid down regulation of ERα expression in cells, was as effective as tamoxifen as a front-line intervention in breast cancer [61]. The first clinically approved molecule of this class, ICI182,780 (fulvestrant) was shown both *in vitro* and in animal models to effectively reduce ERα expression in cells and inhibit the growth of tamoxifen-resistant breast tumor xenografts [62–64]. However, the clinical results with this drug have been extremely disappointing, dampening somewhat the excitement about the potential of this class of molecule [65]. Initially, it was considered that the failure of fulvestrant indicated that the mechanism of tamoxifen resistance modeled in animals did not bear on human tumors. However, it now appears from the results of additional studies that this drug has extremely poor bioavailability and that it is difficult to get high enough levels of the drug at the tumor to effect a quantitative turnover of the receptor [66*]. Indeed, a sequential tumor biopsy study has indicated that even after long-term fulvestrant treatment at the approved dose, ER is still present at approximately 50% of the original baseline [67].Results of recent trials comparing higher doses of fulvestrant to that currently approved demonstrate increased serum steady state levels and correspondingly improved response rate, as well as increased ER turnover, although the pharmaceutical properties of fulvestrant continue to limit its use [65,68,69]. Thus, there is a tremendous amount of interest in developing SERDs that exhibit improved pharmaceutical properties. Interestingly, the compound discussed above, GW5638/DPC974, which by virtue of its effect on ERα structure can disengage cofactors, has been determined to also exhibit SERD activity [70]. Indeed, it has recently been shown that its effect on the structure of helix 12 in ERα is so dramatic that the receptor is recognized as denatured and is targeted for 26S proteasomal degradation. Whereas this molecule itself will not be developed, it has provided a chemical scaffold that can be exploited further for the development of an orally active SERD molecule [71].

Although a detailed discussion of the pharmacology of aromatase inhibitors is beyond the scope of this manuscript, there are several points that are worth mentioning in the context of SERD action. It should be apparent from the studies presented above that inhibition of aromatase and the reduction of circulating/tumor levels of estrogens should result in the inhibition of the growth of ER-positive tumors. However, it should also be clear that the inhibitory actions of these drugs could be bypassed by (a) upregulation of any of the pathways that lead to ligand-independent activation of ERα (i.e. MAPK activation) or (b) the production of, or environmental exposure to, a molecule with estrogen-like properties. Of direct relevance to the former possibility is an important paper published several years ago in which it was shown that exposing breast cancer cells to an aromatase inhibitor resulted in an adaptive change that rendered cells extremely sensitive to low levels of estrogens [72*]. With respect to the latter point is the observation that 27-hydroxycholesterol, an oxysterol produced in a stoichiometric manner from cholesterol in an aromatase-independent manner, can activate ERα and thus could contribute to resistance [73*]. Similarly, it has been shown that the androgen metabolite 3β, 17β-diol can function as an estrogen in cellular models of breast cancer [74]. Clearly, in either of these potential scenarios it would be expected that SERDs would be an effective therapeutic strategy, and indeed fulvestrant was recently shown to have clinical benefit in Her2-overexpressing cancers in patients who had failed endocrine therapy [75].

Final Comments

In the post genome era, there is an abundance of potential new drug targets, a considerable number of which may be relevant to breast cancer. However, there has been a tendency to forget well-validated targets such as ERα in the belief that they have been fully exploited. This could not be further from the truth. It is clear that the more we explore the ERα signal

transduction pathways in breast cancer, the more apparent it is that the existing modulators of this axis are relatively unsophisticated and can be improved. It is also clear also that we have not yet taken full advantage of the complexities in the estrogen signaling pathways in the development of new drugs. However, with a more complete understanding of ER action, as is now emerging, the field is well positioned to move from the standard empirical screening for modulators to specific mechanism-based screens of high predictive value that will likely yield useful drugs.

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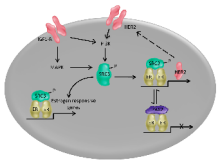

Figure 1. The physical and functional interaction between the ER and Her2/IGFR signaling pathways in breast cancer cells influences the pharmacology of ER ligands

The transcriptional activity of ERα and its pharmacological response to endogenous and exogenous ligands is determined in large part by the repertoire of coregulators expressed in a given cell and the impact of activated signaling pathways on the activity of receptor:coregulator complexes. Whereas there are a large number of coregulators, each of which may have a different effect on receptor activity, a model highlighting the interactions between the ERα-SRC-3/Her2 regulatory axis is presented for illustrative purposes. The complete details supporting this model are presented in the text. In short, however, it is now clear that differences in SRC-3 expression or activity can result in differential activation of the ER target genes. Of particular importance is the observation that increases in SRC-3 activity and or expression can relieve ER-mediated repression of Her2 expression. This initiates a positive feed forward loop that results in increased Her2 signaling and subsequently increased ER signaling. Under these conditions the biocharacter of tamoxifen has been shown to switch from that of an antagonist to an agonist.

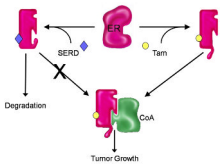

Figure 2. Understanding the role of coregulators in ER action in breast tumors is instructive with respect to new drug development

Upon binding tamoxifen ERα adopts a conformation that is distinct from apo-ERα and that which occurs upon binding estradiol. This conformational change disrupts the primary coregulator binding surface on ERα and reduces the affinity of the receptor for coregulators. Consequently, in cells where coregulators are not overexpressed or hyperactivated, tamoxifen is capable of inhibiting ER action. However, under the selective pressure of tamoxifen administration, something changes within the cell that alters the coactivator milieu such that the tamoxifen:ER complex can engage a coregulator that allows it to activate transcription. This could result from (a) the overexpression of a cofactor with which the receptor normally interacts when occupied by estradiol or (b) the expression of a cofactor that can interact in an ectopic manner with the receptor:tamoxifen complex. The ability of compounds that enable ERα to adopt a unique conformation to effectively inhibit the growth of tamoxifen resistant tumors highlights the validity of this model. It should also be apparent from this discussion why there is so much interest in developing SERDs that function by completely removing the possibility of “productive” ER-coregulator interactions.