Small Molecule Inhibitors as Probes for Estrogen and Androgen Receptor Action

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Abstract:
Since activated estrogen (ER) and androgen receptors (AR) stimulate cell proliferation in breast and prostate cancer, inhibiting their actions represents a major therapeutic goal. Most efforts to modulate ER and AR activity have focused on inhibiting the synthesis of estrogens or androgens, or on identification of small molecules that act by competing with agonist hormones for binding in the ligand-binding pocket of the receptor. An alternative approach is to implement screens for small molecule inhibitors that target other sites in the pathway of steroid receptor action. Many of these second-site inhibitors directly target ER or AR; others have still unknown sites of action. Small molecule inhibitors that target second sites represent new leads with clinical potential, they serve as novel modulators of receptor action and they can reveal new, and as yet unidentified, interactions and pathways that modulate ER and AR action.

Within the large nuclear receptor family, estrogen and androgen receptors are unusual in their ability to stimulate cell proliferation. The central roles played by estrogen receptor α (ERα) and by androgen receptor (AR) in most cases of breast and prostate cancer led to an intense effort to identify agents that modulate receptor activity. The availability of substantial information on the interaction of agonist ligands with ERα and AR led to a primary focus on identification of small molecules that act by competing with natural hormones for binding in the ligand-binding pocket of the receptors. This fruitful approach was followed by development of agents that act by inhibiting key enzymes in estrogen synthesis. While these approaches to development of clinically useful agents remain productive, as shown by recent development of an improved competitive ligand for androgens (1) and an inhibitor of androgen production in prostate tumors (2), their current potential for illuminating novel mechanisms of ER and AR action is limited. Here we focus on small molecules modulators of ER and AR activity that act outside of the ligand-binding pocket. Some of the sites targeted in attempts to antagonize ER action in breast cancer are illustrated in Fig. 1. Research summarized elsewhere in this minireview series describes some of the array of macromolecular interaction partners that can influence receptor activity (3); these include DNA binding sites, proteins that tether receptors to DNA, coactivators and corepressors, chaperones, ubiquitin ligases, and diverse modifiers including kinases, phosphatases, methylases and acetylases. These interactions provide a wealth of targets that are only beginning to be exploited by screens to identify small molecules that modulate ER and AR activity.

In considering efforts to identify small molecule modulators of ER and AR, it is important to understand why it has been simpler to target binding of natural hormone ligands than to target other sites critical for receptor activity. The natural ligands are relatively small, and bind with very high affinity (low nM to sub nM) in a discrete binding pocket whose 3-dimensional structure is known. In contrast, most other ER and AR interactions involve relatively large, often low affinity, macromolecular interfaces for which little or no structural information is available.
available. Most current non-competitive small molecule modulators of ER and AR were identified using screens based on known activities of the receptors, such as DNA binding or coactivator binding. Although binding sites for many of these small molecules are, as yet, unknown, they target well-defined biological processes.

**Small Molecules that Target AR-** The central role of AR in both primary and castration recurrent prostate cancer (CRPC) (4-7) and the limited effectiveness of synthetic AR antagonists, such as hydroxyflutamide and bicalutamide/casodex, that target the ligand-binding pocket of AR, make it an attractive target for development of small molecule inhibitors that target other sites.

More than 350 nuclear receptor coregulators have been described (3,8). The first family of coregulators to be described, the steroid receptor coactivators (SRCs), are ~160,000 kDa proteins containing 3 or 4 Leu-X-X-Leu-Leu (where X is any amino acid) motifs. The SRCs remain among the most important steroid receptor coregulators. SRC3, and the other SRC coregulators, exhibit multiple regulatory functions that go far beyond their interaction with nuclear receptors (3).

In AR and other steroid receptors, agonist binding stabilizes a hydrophobic cleft, termed activation function 2 (AF-2) above the ligand-binding pocket (9,10). In most steroid receptors bound to agonists, including AR, LXXLL motifs in SRC coactivators bind in this hydrophobic cleft. For AR, N/C terminal interaction that results from AR N-terminal FQNLF motif binding to AF-2 competes with binding of coactivator LXXLL motifs (11,12). The coregulator, melanoma antigen gene product (MAGE-11), specifically binds the AR FXXLF motif, thereby increasing accessibility of AF-2 to coactivators. MAGE-11 also directly binds SRC2 and other coactivators, tethering them to AR (13,14). Researchers are only beginning to target the AR-specific interaction surfaces revealed by these studies.

The unique ability of AR to bind larger motifs, such as FXXLF, and WXXVW in phage display libraries (15) suggested an approach to selectively targeting AR. Gunther et al. (16) evaluated a library of coactivator binding inhibitors originally tested on ER (17). Their idea was that pyrimidines containing large aromatic substituents would retain the ability to bind AR, but not ERα. Using a luciferase reporter assay in human endometrial cancer-1 (HEC-1) cells they compared the ability of these compounds to inhibit E2-ERα-dependent expression of an ERE-luciferase reporter and dihydrotestosterone-AR dependent expression of a mouse mammary tumor virus-luciferase (MMTV-Luc) reporter. They also evaluated the compounds activity against the AR T877A mutant found in widely used LNCaP cells and about 30% of patients with metastatic prostate cancer treated with the non-steroidal antagonist hydroxyflutamide (18). Some of the peptidomimetic compounds containing multiple aromatic substituents were highly selective for AR and AR T877A (IC_{50} as low as 2 and 4 μM, respectively) and did not inhibit ERα mediated transactivation (16). Thus, an approach based on side chain size provides a system for producing peptidomimetics that selectively target binding of SRC coactivators to AR, rather than ERα.

In an unusual screen Estebanez-Perpina et al. (19) soaked small molecules into crystals of AR-LBD bound to an SRC fragment and looked for small molecules that disrupted the interaction. They identified a novel hydrophobic binding site, they termed binding function 3 (BF-3). This large site is near AF-2, and is at the junction of the helix H1, and the H3-H5 loop (Fig. 2). Binding of small molecules to this site reorganizes amino acid side chains in both BF-3 and AF-2, resulting in loss of coactivator binding. BF-3 represents a novel allosteric binding site for small molecules that alters AR conformation so that coactivator binding is inhibited. The small molecules identified as binding to BF-3 were quite diverse and include the natural hormone triiodothyronine (T3) (Fig. 2), flufenamic acid and TRIAC. Although the compounds exhibited only modest inhibitory potency (IC_{50} >50 μM) in a fluorescence polarization (FP) assay, they were more effective (IC_{50} 10-30 μM) in reporter gene assays (19). This study is unusual in that detailed structural data of the inhibitor bound to the receptor is available. Although the
concentrations of T3 that bind BF-3 are probably too high to be encountered in biological systems, it remains possible that more potent and selective naturally occurring small molecules allosterically modify coactivator interaction with AR by binding BF-3.

Using a mammalian 2-hybrid screen based on disruption of the interaction of liganded AR with the AR-binding protein, gesolin, Joseph et al. (20) carried out a screen of ~10,000 small molecules and describe 2 structurally distinct compounds, D36 and D80 that inhibit interaction of AR and gesolin. These compounds bind AR at an unknown site outside of the ligand-binding pocket and induce a conformational change that inhibits binding of the synthetic androgen, R1881 and recruitment of AR to androgen-responsive genes. D36 and D80 inhibit transcription of luciferase reporter genes, several endogenous androgen-regulated genes, and androgen-dependent proliferation in cell-based models for anti-androgen-sensitive and anti-androgen resistant prostate cancer with IC50s of 10-40 μM (20).

To identify small molecule inhibitors of AR, Jones et al. (21) used a conformation-based screen based on fluorescence resonance energy transfer (FRET) between cyan and yellow fluorescent proteins fused to AR. Two compounds pyrvinium maoate and harmol hydrochloride inhibited AR activity in the nanomolar range. Pyrvinium and harmol appear to act at distinct unidentified sites outside of the AR ligand-binding pocket. Since they act synergistically and inhibit different steps in AR action, pyrvinium and harmol appear to have different modes of action. Pyrvinium and harmol inhibited the proliferation of LNCaP cells and did not inhibit growth of human embryonic kidney 293 (HEK-293) cells. In a short-term in vivo experiment, pyrvinium combined with bicalutamide caused a 63% reduction in prostate weight (21).

In related work, Jones et al. used the conformation-based FRET screen and high-throughput microscopy to assess nuclear accumulation of AR. The most potent inhibitors targeted pathways known to influence activity of AR and many other proteins (actinomycin [RNA synthesis], cucurbitacin [JAK/STAT3], radicol [HSP90]) (22).

Efforts to target the DNA response element to which AR binds must contend with 2 major issues. There is substantial sequence heterogeneity in naturally occurring AR DNA response elements (AREs). Also, there is a great deal of overlap in DNA binding sites for AR and other receptors. Using a fluorescence polarization/anisotropy microplate assay (FAMA) (23) to analyze binding of purified AR and progesterone receptor (PR) to published DNA-binding-site sequences thought to exhibit some specificity for binding AR over other receptors, we were unable to identify any DNA elements that exhibited a strong preference for binding AR over PR. Dervin and coworkers developed and extensively characterized hairpin polyamides that target the minor groove of the proposed consensus ARE (5'-AGAACAgcaAGTGCT-3') (24). In LNCaP cells treated with 10 μM polyamide, binding of AR to androgen response elements was reduced and androgen induction of the prostate specific antigen (PSA) and FKBP5 genes was inhibited by 60-70%. Analysis of the effects of the polyamide on the entire set of transcripts in LNCaP cells suggested that the heterogeneity of the AR DNA binding sites, and the existence of AR genes regulated by indirect mechanisms, will make it impossible for a single polyamide to inhibit all AR-regulated genes (24). However, these compounds may have considerable potential in studies designed to inhibit genes containing specific AREs, or families of closely related AREs.

Small Molecules that Inhibit ER-coregulator Interactions- Most early efforts to identify small molecule that interfere with ER activity and act outside of the ligand binding pocket focused on compounds that interfere with coactivator binding (Fig. 1). Katzenellenbogen and coworkers used a fluorescence polarization assay to identify pyrimidines that inhibit binding of a coactivator peptide from SRC1 to ERα (25). To optimize these compounds, they synthesized a larger pyrimidine-based library (17) and evaluated the compounds using time-resolved FRET (26). Further studies showed these compounds inhibited ERα-mediated activation of a transiently transfected luciferase reporter gene. A different chemical scaffold based on
amphipathic benzenes led to inhibitors with mean inhibitory concentrations of 1.7 μM (27).

Using high throughput screening for inhibitors of ER-coactivator interaction, the guanylhydrazone ERI-05 was identified. In mammalian 2-hybrid assays, ERI-05 inhibited interaction of the Gal4 DNA binding domain-ERα ligand binding domain (GalDBD-ERLBD) fusion and SRC1, SRC2 and SRC3-VP16 fusions with an IC50 of 6 μM. However, the 20 μM concentration of ERI-05 required to inhibit ERα-induction of the endogenous pS2 gene in MCF-7, human breast cancer cells, approaches the concentration that is toxic to cells (28). Development of these compounds continues and some show improved potency relative to ERI-05 (29).

In the classical model for antagonist action, binding of 4-hydroxytamoxifen (OHT) to ER results in a conformation in which a segment of helix 12 of the ER occupies the coactivator binding groove (30). In an intriguing structural study of the ERβ ligand-binding domain, a second molecule of OHT was bound to the receptor and occupied the hydrophobic groove of the coactivator binding surface (31). Whether this second binding site plays a direct role in OHT antagonism of coactivator binding, and the level of OHT required to occupy this site, remain to be established.

Although they are not small molecules, coactivator peptide inhibitors displayed a novel mechanism for inhibition of ER. A nona-arginine TAT peptide tag, to improve cell permeability, linked to the SRC2/TIF2 box 2 peptide, resulted in an inhibitory peptide that at 70 μM completely blocked E2-ERα-induction of pS2 mRNA in MCF-7 cells (32). In addition to facilitating passage across the plasma membrane, linking peptides to oligo-arginine results in their accumulation in the nucleolus. Using an ERα-cyan fluorescence protein fusion transfected into U2OS cells enabled Carraz et al. (32) to show that binding of these peptides to ERα results in sequestration of ERα into the nucleolus. This is an example of a second-site inhibitor that acts in part by altering sub-cellular localization of a steroid receptor.
[(benzylthio)methyl]- (TPBM) (Fig. 3A) was selected for further study (37). TPBM preferentially inhibited binding of ER to the cERE (IC_{50}s for binding to ER{\alpha}, PR and AR of 3, 10 and 8 {\mu}M, respectively). Since we found that increasing the concentration of E_{2} in the binding assays to 10 {\mu}M had no effect on the ability of TPBM to inhibit binding of ER{\alpha} to the flcERE, TPBM does not act by binding in the ligand-binding pocket of ER{\alpha}. Unlike DIBA, TPBM does not act as a zinc chelator. In stably transfected cell lines expressing a luciferase reporter linked to EREs, or to the PR and GR-regulated MMTV PRE, TPBM inhibited expression of the estrogen responsive reporter responsive with an IC_{50} of 12 {\mu}M and did not inhibit PR and GR-regulated transcription (37).

To analyze the intracellular action of TPBM, we examined its ability to inhibit expression of the estrogen-inducible proteinase inhibitor-9 (PI-9) gene. Induction of the serpin and tumor lethality factor PI-9 results from direct binding of E_{2}-ER{\alpha} to EREs and ERE half sites (38,39). Estrogen induction of PI-9 enables breast cancer cells to evade apoptosis induced by immune cells (40,41). We used MCF7ER{\alpha}HA cells, which overexpress ER{\alpha} (42). In these cells, tamoxifen and 4-hydroxytamoxifen (OHT) are potent agonists (40,43). TPBM inhibited both E_{2} induction of PI-9 mRNA (IC_{50} 9 {\mu}M) and OHT-induction of the PI-9 gene. Using quantitative RT-PCR to measure the PI-9 mRNA level and semi-quantitative ChIP to measure occupancy of the PI-9 estrogen responsive region, we observed a good correlation between the extent to which TPBM inhibits induction of PI-9 mRNA and the extent to which TPBM reduces E_{2}-ER{\alpha} occupancy at the PI-9 gene (37). Thus, the primary mechanism by which TPBM exerts its intracellular action is by decreasing interaction of ER{\alpha} with regulatory regions of estrogen-responsive genes. It is likely that interaction of TPBM with ER{\alpha} induces a conformational change in the receptor, and that one result of this conformational change is decreased association of E_{2}-ER{\alpha} with EREs. TPBM is a useful research tool to probe the role of DNA binding in ER-mediated processes.

TPBM has been used to inhibit binding of ER to EREs in several genes (44,45) and to analyze the contribution of DNA binding to stabilization of ER dimers (46).

To identify more potent ER{\alpha} inhibitors, we used a cell-based screen to evaluate ~200 small molecules structurally related to TPBM and identified a much more effective ER inhibitor, butyrophenone, p-fluoro-4-(1,2,3,6-tetrahydro-1,3-dimethyl-2-oxo-6-thionpurin-8-ylthio) (TPSF) (Fig. 3A) (47).

**Down-regulation of ER{\alpha} Levels by a Small Molecule Inhibitor**

- **In testing using ER{\alpha} positive T47D, human breast cancer cells, stably transfected to express an (ERE)-luciferase reporter, TPSF with an IC_{50} of 0.7 {\mu}M was ~15 fold more potent than TPBM. Competitive radiometric binding assays and cell-based inhibition studies done at E_{2} concentrations that vary by 500 fold demonstrate that TPSF is not a classical ligand that competes with E_{2} for binding in the ER{\alpha} ligand-binding pocket.**

  - In MCF-7 cells, TPSF potently inhibits E_{2}-ER{\alpha}-mediated induction of the PI-9 gene (IC_{50} 200 nM), which is activated by direct binding of ER{\alpha} to an estrogen response element DNA and the cyclin D1 gene, which is thought to be induced by tethering ER{\alpha} to other DNA-bound proteins. Since 30 {\mu}M TPSF had no effect on the NF-\kappaB-mediated induction of IL-8 mRNA in MCF-7 cells, and very high concentrations of TPSF were required to inhibit GR and AR-mediated transcription, TPSF is a selective inhibitor of ER{\alpha} (47).

  - We tested TPSF inhibition of cell growth in ER{\alpha}-positive MCF-7 cells and in ER{\alpha} negative MDA-MB-231 human breast cancer cells. TPSF elicited a dose-dependent inhibition of the estrogen-dependent growth of MCF-7 cells with an IC_{50} of 2 {\mu}M and completely blocked estrogen-dependent growth by 7.5 {\mu}M. In the ER-negative MDA-MB-231 cells, TPSF did not inhibit growth at all concentrations, including 30 {\mu}M. The capacity for anchorage-independent growth is a hallmark of cancer cells that is often evaluated by growth in soft agar. MCF-7 cells grown in medium containing E_{2} formed large colonies. The addition of 10 {\mu}M TPSF completely blocked formation of MCF-7 cell colonies (47). Thus, TPSF inhibits estrogen-stimulation of both...
anchorage-dependent and anchorage-independent growth of breast cancer cells.

TPSF also inhibits ERα-dependent cell growth in 3 models for tamoxifen resistance: 4-hydroxytamoxifen-stimulated MCF7ERαHA cells that overexpress ERα, in fully tamoxifen-resistant BT474 cells that have amplified HER-2 and AIB1, and in partially tamoxifen-resistant ZR-75 cells. Thus, TPSF is effective in cells that become tamoxifen-resistant through different mechanisms.

Although TPBM and TPSF are very similar structurally (Fig. 3A), they have very different effects on ERα. While TPBM inhibits in vitro binding of E2-ERα to a labeled ERE in vitro, TPSF does not (Fig. 3B). TPSF strongly reduces ERα levels in breast cancer cells, while TPBM has little or no effect on the level of ERα (Fig. 3C). Since TPSF has very little or no effect on the levels of AR and GR, TPSF is highly selective for down-regulation of ER. The proteasome inhibitor MG132 abolished down-regulation of ERα by TPSF (47). Thus, TPSF influences receptor levels at least in part due to its ability to enhance proteasome-dependent degradation of ERα.

How might we account for the different modes of action of these two closely related small molecules? ERα and other steroid receptors exhibit a high level of conformational flexibility and small molecules can elicit quite different conformations when they interact with ERα. For example, binding of E2 or OHT in the ERα ligand-binding pocket results in functionally distinct agonist and antagonist conformations (30). Thus, binding of TPBM, and the more potent TPSF, may cause distinct ERα conformations that are associated with different modes of action. Our observation that TPSF down-regulates E2-ERα more effectively than unliganded ERα is consistent with an important role for receptor conformation in TPSF action.

Although several pathways modulate ERα degradation, mechanisms by which a small molecule might enhance degradation of ERα are poorly defined. Activation of the aryl hydrocarbon receptor (AhR) by the dioxin TCDD stimulates proteasome-dependent degradation of ERα (48). Knockdown of the oncprotein Muc1, which binds to and stabilizes ERα, increases ERα degradation and inhibits ERα-mediated transactivation and growth of breast cancer cells (49). Fulvestrant/faslodex/ICI 182,780 is a high affinity antagonist ER ligand used therapeutically to treat advanced breast cancer that enhances degradation of ERα (50,51). Recent structural studies suggest fulvestrant binding may distort ERα structure so that a few hydrophobic amino acids are exposed near the surface, perhaps triggering recognition of ERα as a misfolded protein and rapid degradation.

**Future Prospects** - The use of small molecules identified by physical or virtual screening to illuminate the actions of steroid receptors is gaining momentum. While most studies have focused on small molecules that act at known well-defined sites in steroid receptor action, an intriguing approach, that is still in its infancy, is to use cell and organism-based high throughput screens based on inhibition of receptor activity or cell growth. Cell and organism based screens can potentially identify any small molecule whose actions influence receptor-mediated transactivation or cell growth. This type of unbiased screen asks the cell to tell us what interactions and pathways are susceptible to targeting by small molecules with a read-out of altered receptor-mediated transactivation or cell proliferation. Small molecule inhibitors identified using cell-based screens have the potential to identify novel pathways and interactions that influence receptor activity. By their very nature it is likely to be both challenging and rewarding to identify these novel sites of inhibitor action.
REFERENCES

FOOTNOTES

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1M. Cherian and D. Shapiro, unpublished observations

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Abbreviations: ERα, estrogen receptor α; E2, 17β-estradiol; FAMA, fluorescence anisotropy microplate assay; TPSF, butyrophenone, p-fluoro-4-(1,2,3,6-tetrahydro-1,3-dimethyl-2-oxo-6-thionpurin-8-ythio); TPBM, 8-benzylsulfanylmethyl-1,3-dimethyl-3,7-dihydro-purine-2,6-dione; OHT, 4-hydroxytamoxifen; PSA, prostate specific antigen; DMSO, dimethyl sulfoxide; AR, androgen receptor; GR, glucocorticoid receptor; ERE, estrogen response element; AIB1, amplified in breast cancer 1; IC_{50}, inhibitor concentration for 50% inhibition; IL-8, interleukin 8; qRT-PCR, quantitative reverse transcriptase-PCR.
FIGURE LEGENDS

Fig. 1. Schematic representation of some sites targeted by small molecules used to selectively block ERα action and breast cancer cell growth. Current small molecule modulators largely target estrogen synthesis and competition with 17β-estradiol for binding in the ligand-binding pocket of ERα. Actions of ERα targeted by the small molecules discussed here included coactivator binding, DNA binding and receptor degradation.

Fig. 2. Structure of the AR-LBD with T₃ bound to AR-BF3. Ribbon diagram of AR ligand-binding domain, liganded with DHT (brown) and triiodothyronine T3 (purple) bound at BF-3. A dot-filling model illustrates the residues in the AF-2 core. (Prepared using Jmol (http://www.jmol.org/) from PDB accession code 2PIV).

Fig. 3. Structurally similar TPBM and TPSF have very different modes of action. A. Structures of the ERα inhibitors TPBM and TPSF. B. In contrast to TPBM, TPSF does not inhibit binding of E₂-ER to a fluorescein-labeled cERE. C. Western blot analysis shows that TPSF elicits a concentration-dependent decline in ERα levels. In contrast, TPBM has no effect on effect on the level of ERα (redrawn from (47)).
Figure 1:
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