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Differential Presentation of Protein Interaction Surfaces on the Androgen Receptor Defines the Pharmacological Actions of Bound Ligands

John David Norris,[1,5,6] James David Joseph,[1,5,6] Andrea Barreto Sherk,[1] Dalia Juzumiene,[1,3] Philip Stewart Turnbull,[2] Stephen William Rafferty,[2] Huaxia Cui,[1] Erin Anderson,[1] Daju Fan,[1] Delita Arnelle Dye,[1,2] Xiang Deng,[1,4] Dmitri Kazmin,[1] Ching-Yi Chang,[1] Timothy Mark Willson,[2] and Donald Patrick McDonnell[1,*]

[1]Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC 27710, USA
[2]GlaxoSmithKline, Five Moore Drive, Research Triangle Park, NC 27709-3398, USA
[3]Affinergy Inc., 617 Davis Drive, Suite 100, Durham, NC 27713, USA
[4]Core Genotyping Facility, Advanced Technology Program, SAIC Frederick, Inc., NCI-Frederick, Frederick, MD 21702, USA
[5]These authors contributed equally to this work
[6]Present address: Integrated Oncology Solutions Inc., 7020 Kit Creek Road, Suite 130, P.O. Box 13399, Research Triangle Park, NC 27709, USA
*Correspondence: donald.mcdonnell@duke.edu
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SUMMARY

The pharmacological activity of different nuclear receptor ligands is reflected by their impact on receptor structure. Thus, we asked whether differential presentation of protein-protein interaction surfaces on the androgen receptor (AR), a surrogate assay of receptor conformation, could be used in a prospective manner to define the pharmacological activity of bound ligands. To this end, we identified over 150 proteins/polypeptides whose ability to interact with AR is influenced in a differential manner by ligand binding. The most discriminatory of these protein-AR interactions were used to develop a robust compound-profiling tool that enabled the separation of ligands into functionally distinguishable classes. Importantly, the ligands within each class exhibited similar pharmacological activities, a result that highlights the relationship between receptor structure and activity and provides direction for the discovery of novel AR modulators.

INTRODUCTION

The steroid receptor subfamily of ligand-regulated transcription factors comprises well-validated drug targets, modulators of which are used extensively in the clinic to manipulate normal endocrine signaling or to block inappropriate cellular responses to specific hormones. Until recently, the most pharmaceutically important modulators of these receptors were agonists that mimicked the actions of physiological hormones or antagonists that opposed the actions of endogenous activating ligands. However, there has been a significant paradigm shift in this area of drug discovery fuelled by the observation that the complex signaling pathways regulated by steroid hormones can be exploited to yield compounds that manifest cell and/or process selectivity. The practical impact of this observation was highlighted by a seminal study demonstrating that tamoxifen, an antiestrogen in breast, actually functioned as an estrogen in the skeletal system (Love et al., 1992). Thus, it appeared that tamoxifen, rather than functioning as an antagonist in all tissues, is actually a selective estrogen receptor modulator (SERM), a compound whose relative agonist/antagonist activities are manifest in a cell-selective manner (Sato et al., 1996). Subsequently, other SERMs, like raloxifene, have been developed for the treatment and prevention of postmenopausal osteoporosis (Cole et al., 1998). However, the observation that tamoxifen, and now raloxifene, also significantly reduce the incidence of breast cancer in patients at elevated risk for the disease has clearly demonstrated the benefit of developing compounds with dual agonist/antagonist activities (Vogel et al., 2006). The clinically favorable profile of this new class of drug has reinvigorated the field with the anticipation that, by understanding their mechanism of action, it will be possible to develop SERMs with more useful therapeutic activities. In addition, the clinical success of SERMs has resulted in a heightened level of interest in developing selective modulators of the progesterone, mineralocorticoid, glucocorticoid, and androgen receptors for the treatment of a variety of different endocrinopathies.

Our interests have focused recently on the development and application of mechanism-based approaches to discover new classes of androgen receptor (AR) modulators. Androgens are key regulators of processes involved in the development and maintenance of normal reproductive function in males and also exert significant anabolic actions in both bone and skeletal muscle. Not surprisingly, therefore, in addition to the treatment of hypogonadism, there is a high level of interest in using androgens for the treatment of sarcopenia, osteoporosis, and muscle wasting associated with certain diseases (including cancer and AIDS) (Cadilla and Turnbull, 2006; Negro-Vilar, 1999). However, androgens and aberrant AR signaling have also been implicated in the pathology of benign prostatic hypertrophy and prostate cancer, raising the concern that chronic administration of AR agonists might have a negative effect on prostate health. Thus,

in addition to the obvious need for antiandrogens in the treatment (and possible prevention) of prostate cancer, there is an unmet medical need for AR ligands that exhibit anabolic activity in muscle and bone but have reduced activity in the prostate. Several drugs with these general characteristics, comprising a subclass of AR ligands called selective androgen receptor modulators (SARMs), have been identified and are currently being evaluated in the clinic for a variety of conditions (Gao and Dalton, 2007). However, the molecular basis for the selectivity of this class of drugs has not been established, and it is not clear if they represent the optimal modulators of AR signaling for clinical use. For this reason, there is a need to understand the molecular mechanisms that determine the pharmacological activity of AR ligands to direct the discovery of the next generation of process-specific modulators.

Much of what is known about the molecular determinants of nuclear receptor (NR) pharmacology has come from the study of the SERMs tamoxifen and raloxifene. Using differential sensitivity to proteases, it was shown that these SERMs induced a conformational change in estrogen receptor alpha (ERα) that was distinct from that observed when the receptor was occupied by either agonists or pure antagonists (McDonnell et al., 1995). Subsequent crystallographic analysis of the isolated hormone binding domain of ERα occupied with 17β-estradiol, or with different SERMs, confirmed these alterations in receptor structure and mapped a major conformational change to the AF-2 coactivator interaction domain located within the carboxyl terminus of the receptor (Brzozowski et al., 1997; Shiau et al., 1998; Wu et al., 2005). Studies using combinatorial peptide phage display highlighted the dynamic, flexible nature of the AF-2 pocket and how this was influenced by different SERMs (Connor et al., 2001; Norris et al., 1999). However, although these studies of ER structure and AF-2 architecture revealed how SERMs functioned as antagonists in some contexts, they did not explain the dramatic functional differences exhibited by tamoxifen and raloxifene in the reproductive system. This raised the possibility that protein-protein interaction surfaces, in addition to AF-2, are presented on the surface of SERM-activated ER, allowing it to engage cofactors that promote agonist activity in some tissues. Indeed, this idea is supported by mutagenesis studies showing that domains other than AF-2 are required for SERM agonist activity (Tzukerman et al., 1994).

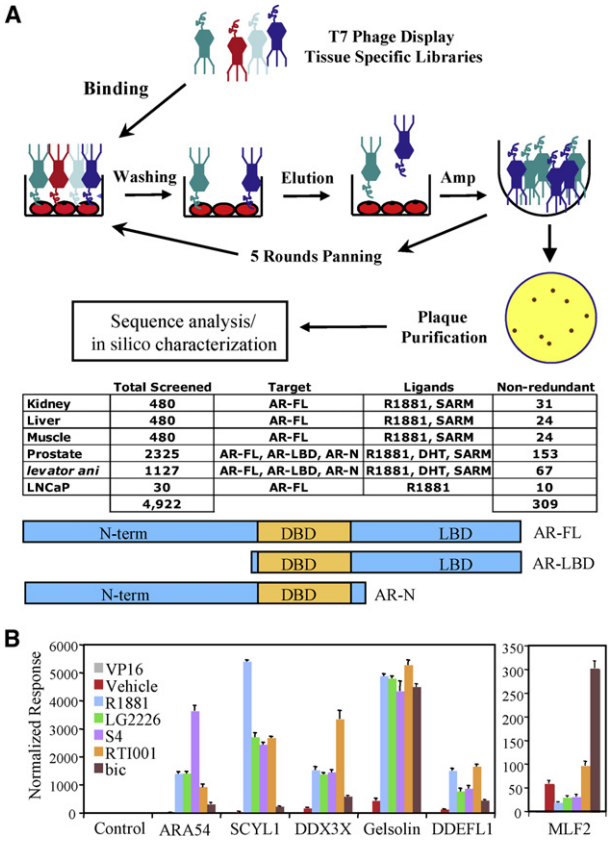
Although crystallography and combinatorial peptide phage display have been useful in understanding the role of agonists and antagonists in the presentation of the AF-2 cofactor-binding pocket within AR (Chang and McDonnell, 2005; Matias et al., 2000; Ostrowski et al., 2007; Wang et al., 2006), neither approach has been informative with respect to the impact of ligands on the presentation of surfaces, other than AF-2, that are likely to be involved in cofactor binding. This is somewhat surprising because there is a significant amount of biochemical data to suggest that AR function is influenced by ligand-regulated intradomain interactions (He et al., 2000; He and Wilson, 2002). Thus, it is likely that structurally complex, protein-protein interaction surfaces are lost in studies of isolated domains. This problem might also apply to the use of short peptide probes to study AR structure, because they might be unable to interact with complex binding surfaces that require intradomain interactions. To overcome these limitations, we have used T7 phage display to identify proteins/peptides whose interaction with full-length AR is influenced by the nature of the bound ligand. Although it is unlikely that all of the proteins/polypeptides identified are derived from physiologically relevant AR cofactors, we have shown that they provide sufficient structural complexity to enable an evaluation of the role of ligands in regulating the presentation of different protein-protein interaction surfaces on AR. Furthermore, we have demonstrated that the differential presentation of protein-protein interaction surfaces by AR ligands can be used in a prospective manner to predict their inherent pharmacological activities.

RESULTS

Identification of AR-Interacting Proteins

AR is a large, multidomain transcription factor whose functional activity requires both intramolecular interactions between receptor domains and intermolecular interactions with cofactors and DNA (Chang and McDonnell, 2005; He and Wilson, 2002). Given the difficulty of defining multiple protein-protein interaction surfaces on the receptor using combinatorial peptide phage display (Norris et al., 1999), we reasoned that many of the surfaces on AR that are required for activity are diffuse and complex. Thus, the interaction domain(s) of some associated proteins are likely to be relatively large and would not be expected to be identifiable using small peptides. Consequently, we initiated a project directed toward the identification of intact proteins (or protein fragments) that could be used to survey the protein-protein interaction surfaces presented on ligand-activated AR. To this end, high-throughput T7 phage display technology was used to screen cDNA expression libraries derived from androgen responsive tissues and cell lines (human liver, human kidney, human muscle, human prostate, LNCaP, and rat *levator ani*) for proteins that interact in a specific manner with ligand-bound AR (Figure 1A). Although the identification of protein probes of AR structure was the primary goal of this project, we reasoned that by using phage libraries derived from AR-expressing cells we could also identify functionally relevant cofactors.

For these screens, recombinant biotinylated AR (full-length AR, amino acids [aa] 1–919; AR-LBD, aa 507–919; and AR N-term, aa 1–660) (Figure 1A) was purified from *Spodoptera frugiperda* cells in the presence of either agonists (R1881, dihydrotestosterone [DHT]) or SARMs (LG2226, S4, GW579, GW980) (Juzumiene et al., 2005) and immobilized to 96 well plates using the C3 androgen response element (ARE) (Kallio et al., 1994). The immobilized AR was used as bait in screens for T7 phage expressing AR-interacting proteins. Prior to screening, we confirmed that biotinylation of AR did not have a negative impact on its function by demonstrating that this modified form of the receptor was transcriptionally active in mammalian cells (not shown). Following sequence analysis and in silico characterization of the cDNA inserts from more than 4900 purified phage, we identified 309 nonredundant clones whose expressed products interacted with AR (Figure 1A). The identity of the proteins corresponding to each clone and additional details of the screen are presented in Table S1 (available online). Several previously identified AR- and NR-interacting proteins were identified in this screen including ARA24, gelsolin, PTEN, TFIIF, supervillin, HOXB13,



	Total Screened	Target	Ligands	Non-redundant
Kidney	480	AR-FL	R1881, SARM	31
Liver	480	AR-FL	R1881, SARM	24
Muscle	480	AR-FL	R1881, SARM	24
Prostate	2325	AR-FL, AR-LBD, AR-N	R1881, DHT, SARM	153
levator ani	1127	AR-FL, AR-LBD, AR-N	R1881, DHT, SARM	67
LNCaP	30	AR-FL	R1881	10
	4,922			309

Figure 1. Identification of AR-Interacting Proteins Using T7 Phage Display

(A) Scheme used for affinity selection of AR-interacting proteins. Tissue specific T7 phage display libraries (human prostate, human muscle, human kidney, human liver, LNCaP, and rat *levator ani*) were incubated with AR target protein (AR-FL, aa 1–919; AR-LBD, aa 507–919; and AR N-term, aa 1–660) bound by ligand (R1881, DHT, SARM). Unbound phage were removed by washing and bound phage were eluted and amplified. The process was repeated for a total of five rounds of panning. Individual phage were plaque purified and identity of AR-interacting protein was deduced by DNA sequencing. A summary of the screening results is shown in the table (inset). In total, 4922 individual phage were sequenced, resulting in 309 nonredundant clones (Table S1).

(B) A mammalian two-hybrid assay was used to evaluate ligand-dependent recruitment of selected T7 clones with AR in cells. HepG2 cells were transfected with AR-VP16 and indicated T7 clone (expressed as a fusion with the Gal4 DNA binding domain) along with the Gal4- responsive reporter gene (5xGalLuc3) and pCMV-βGal. Cells were induced for 48 hr with indicated ligand (100 nM for all ligands except bicalutamide [bic], which was used at 1 uM). Data are presented as normalized response, which was obtained by normalizing luciferase activity to β-galactosidase activity. ARA54, positive control; zsGreen, negative control; VP16, empty VP16 vector in place of AR-VP16. The data are representative of three separate experiments. The error bars represent the standard deviation of a single experiment performed in triplicate.

TRIP12, and PPARBP (Hsiao et al., 1999; Jung et al., 2004; Lee et al., 1995; Lin et al., 2004; McEwan and Gustafsson, 1997; Ting et al., 2002; Wang et al., 2002). The identification of multiple alleles of known AR- and NR-interacting proteins served as an initial validation of the approach we used to identify protein domains that were capable of highlighting functionally important protein-protein interactions surfaces on the receptor. Importantly, several proteins were identified that interact with the N terminus of AR and are thus independent of AF-2 (Table S1).

A gene ontology analysis of the primary AR interactors indicated that several protein classes were overrepresented in this collection compared with a reference protein module using ONTO-tools (Khatri et al., 2004). As expected, proteins involved in gene expression and nucleotide binding were the most overrepresented, followed by proteins involved in metal ion binding and cytoskeletal interactions (see Figure S1). Interestingly, the previously defined cofactors that contain the NR- interaction motifs LXXLL (Chang et al., 1999; Heery et al., 1997) and FXXLF (AR-cofactors) (He et al., 2000, 2002) were underrepresented in the proteins identified in our screens. The specific motifs that enable the interaction of the proteins identified with AR remain to be defined.

The ability of each clone, identified in the primary screen, to interact with AR in cells was examined using a mammalian two-hybrid assay. Specifically, each clone, expressed as a fusion protein with the Gal4 DNA binding domain (DBD), was tested for its ability to interact with full-length AR-VP16 in the presence of either R1881 or a SARM. Of the 302 clones tested in this manner, 162 were found to interact with AR and were brought forward for further analysis (Table S1).

Classification of AR-Interacting Proteins Based on Their Ability to Associate with Different Receptor-Ligand Complexes

One of the assumptions underlying AR pharmacology is that ligands regulate the presentation of different protein-protein interaction surfaces on AR. Thus, we assessed the ability of each of the 162 proteins identified to interact with AR in the presence of pharmacologically distinct ligands using a mammalian two-hybrid assay with a view to separating these proteins into functionally distinct groups. Representative data highlighting the impact of ligands on the interaction of AR with selected proteins are presented in Figure 1B. Interestingly, the previously identified AR coactivator, ARA54 (Kang et al., 1999), interacts with AR when bound by all of the ligands tested. However, the interaction is significantly enhanced in the presence of the SARM S4 (Yin et al., 2003). SCYL1 exhibits similar preferences as ARA54, although in this case R1881-activated AR yields the most robust response. Both DDX3X (a DEAD box RNA helicase) and DDELF1 (development and differentiation enhancing factor-like 1) interact with RTI001-activated AR, with DDELF1 also demonstrating a preference for R1881-bound receptor. Gelsolin, a previously identified AR coregulatory protein (Nishimura et al., 2003), interacts with AR in the presence of all ligands tested including the antagonist bicalutamide. We also identified several proteins, like MLF2, that interact with apo-AR but whose interaction is significantly diminished in the presence of agonists and enhanced by bicalutamide. A similar analysis was performed with each of the 162 confirmed AR interactors. At the conclusion of these experiments, we were able to define eight distinct classes of ligand-modulated interaction profiles that best described the receptor binding characteristics of the 162 interactors. For example, the interaction profiles of proteins in the same class as ARA54, as described in Figure 1B, were modulated similarly by ligands (S4 > R1881 ≈ LG2226 > RTI-001 > bicalutamide). These groups were based solely on the interaction

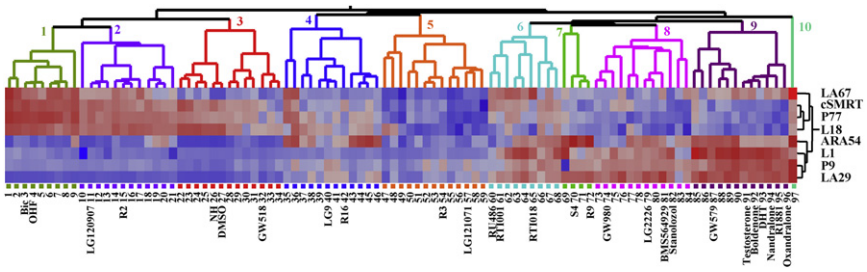


Figure 2. Differential Cofactor Recruitment Defines Distinct Classes of AR Ligands

Mammalian two-hybrid assay was performed as in Figure 1B except that Renilla-luciferase was used to normalize for transfection efficiency. Interaction profiles of 95 androgen receptor ligands and vehicle controls were generated using eight cofactors. The profiles were analyzed with the Ward hierarchical cluster algorithm using standardized data. The resulting dendrogram and structural activity heatmap demonstrates the relationships between the ten structure-based clusters. The 25 labeled compounds are AR ligands with previously characterized biological activities. Controls are indicated as DMSO and NH, reflecting the vehicle alone or no vehicle addition.

profile and were derived without regard to the strength of interaction. Subsequently, we selected a single, representative protein/peptide probe from each class, for inclusion in the profiling tool described below. It is important to note that although we identified potentially interesting AR-interacting proteins in this screen, it is their ability to survey the presentation of different protein-protein interaction surfaces on AR following activation by different ligands that is the focus of the current study. Thus, the next step was to determine the extent to which protein-protein interaction profiles could be used to predict the pharmacological activity of AR ligands.

AR Ligands Can Be Distinguished Based on Their Ability to Present Different Protein-Protein Interaction Surfaces on the Receptor

The diverse set of AR interacting proteins we identified afforded us the opportunity to apply a chemical-biological approach to address the relationship between the presentation of different protein-protein interaction surfaces and the pharmacological activity of ligands. Specifically, we screened a library of structurally diverse AR ligands and assessed their ability to differentially regulate the interaction of AR with a representative protein probe from each of the eight groups highlighted by the preliminary studies described above. The set of compounds chosen for this analysis includes a number of well-characterized AR ligands; (a) antagonists (bicalutamide, hydroxyflutamide [OHF], and LG120907; Hamann et al., 1998), (b) full agonists (R1881 and DHT), and (c) SARMs (S4, LG2226, and BMS564929) (Gao and Dalton, 2007; Miner et al., 2007; Ostrowski et al., 2007; Yin et al., 2003). We also included a large number of novel ligands that we identified for which no biology is known but had a K_i of less than 1.0 μM in AR binding assays (Table S2). The results of this screen are presented in Figure 2.

To avoid signal strength bias, the interaction data for each individual protein was standardized. The individual interaction profiles were then clustered by hierarchical analysis using the Ward hierarchical cluster algorithm (Ward, 1963). Ligand cluster 1 contains OHF and bicalutamide and thus represents the interaction profile of an antagonist. Cluster 2 contains another group of antagonists (or very weak agonists), LG120907 and R2 (Dalton et al., 1998). Cluster 3 represents the protein binding profile of the receptor in the presence of dimethyl sulfoxide (DMSO) or in the absence of any compound (apo-receptor). Interestingly, we identified compounds in this cluster that interact with AR, but whose protein binding profiles are indistinguishable from the unliganded receptor. We predict that compounds in this cluster could represent pure competitive antagonists that bind and freeze the receptor into an ''apo'' conformation. Indeed, cluster 3 ligands were found to inhibit R881-mediated AR transcriptional activity (Figure S2). Clusters 4 and 5 represent compounds that display varying degrees of partial agonist properties. Cluster 6 contains most of the RTI series of RU486-derived AR ligands (Sathya et al., 2003). Both RTI001 and RTI018 are found in this cluster, although they reside in different nodes, a reflection, possibly, of the subtle differences in the pharmacological activities of these ligands that we have observed previously (Kazmin et al., 2006; Sathya et al., 2003). This cluster also contains unrelated nonsteroidal compounds, demonstrating that the peptide interaction profile likely reflects structural changes in AR, rather than being related to a particular chemotype. Cluster 7 contains S4, a compound that has previously been shown to have SARM activity (Yin et al., 2003). Among the compounds in cluster 8 are additional SARMs LG2226 and BMS564909, both of which exhibit prostate sparing properties (Gao and Dalton, 2007; Miner et al., 2007; Ostrowski et al., 2007; Yin et al., 2003). Cluster 9 contains DHT and testosterone (T) and is populated by most of the well-studied AR agonists and anabolic steroids. Finally, cluster 10 contains a single compound with superagonist properties. Note that the clusters represent distinct protein/peptide interaction profiles and are not a continuum that represents a global increase or decrease in the interaction with the selected probes.

Defining the Relationship between Differential Presentation of Protein-Protein Interaction Surfaces and Biological Response

To probe the relationship between the presentation of different protein-protein interactions surfaces and the pharmacological activity of bound ligands, we first tested the activity of all 95 compounds in a transient transfection assay using an AR-responsive mouse mammary tumor virus (MMTV)-luciferase reporter. The data from this analysis, presented in Figure 3A, have been ordered and colored to distinguish the various clusters generated by the protein interaction studies. Although not absolute, it is apparent that the relative transcriptional activity of the individual ligands increases from inactive to active as the cluster numbers increase. A principal component (PC) analysis was performed on the dataset presented in Figure 2, and the first three principal

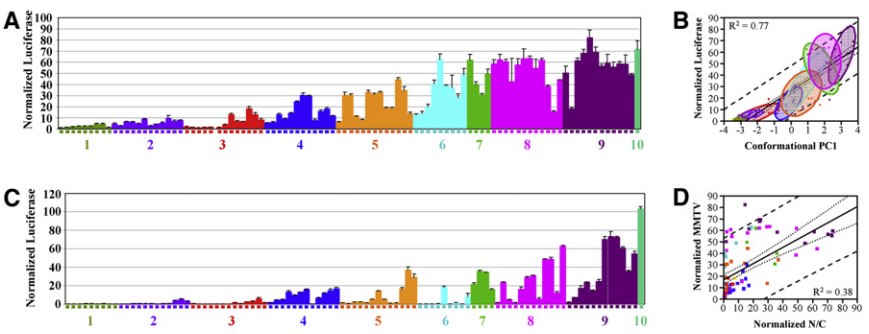


Figure 3. Relationship between Ligand-Induced Conformation and AR Transcriptional Activity

(A) Transcriptional activity of all 95 compounds from Figure 2 was determined in HepG2 cells using a MMTV-luciferase reporter assay. Compounds are presented in the same order and colored by conformation-based clustering as in Figure 2.

(B) Correlation between AR MMTV-luciferase activity and conformation. Principle component analysis was performed using the conformational data presented in Figure 2. Principle component 1 was plotted versus MMTV-luciferase activity. Data are plotted as 75% density ellipses in the color corresponding to the clusters. The dotted line represents the 95% confidence interval, whereas the dashed line represents the 95% prediction interval.

(C) The ability of all 95 compounds from Figure 2 to facilitate the AR N/C-terminal interaction was determined in HepG2 cells. Data are presented as described for MMTV-luciferase activity in (A).

(D) The correlation between AR N/C-terminal interaction and MMTV-luciferase activity. Compounds are colored according to their conformation cluster.

Data presented in (A) and (C) are representative of two independent experiments. The error bars represent the standard deviation of a single experiment performed in triplicate.

components were plotted against the relative transcriptional activity of every compound to determine the extent to which the protein interaction profile correlates with transcriptional activity. Presented in Figure 3B is the MMTV-luciferase transcriptional activity plotted against the conformational PC1. As can be seen, there is a clear correlation between PC1 and relative MMTV transcriptional activity (R^2 = 0.77). These data suggest that the protein interaction profiles identified are predictive of the relative agonist, partial agonist, and antagonist activity of compounds, as assessed using a simple transcriptional assay. Previously, it has been reported that the ability of compounds to facilitate an interaction between the amino and carboxyl termini of AR is a good predictor of agonist efficacy (Kemppainen et al., 1999). However, we were unable to establish a strong correlation between agonist activity and N/C-terminal interaction in our studies. Cluster 8, for instance, is populated with agonists, whereas only a subset of these compounds is capable of promoting N/C-terminal interactions (Figure 3C). Similar results can be seen with compounds from clusters 5 and 6. When all compounds are considered, we find only a weak correlation between activity in the N/C-terminal interaction assay and MMTV transcription (R^2 = 0.38) (Figure 3D).

The next step was to determine whether the correlation between the protein-protein interaction profiles and the transcriptional activity observed in the transient transfection assays persisted when an analogous study was performed using endogenous genes in LaPC4 prostate cancer cells (Klein et al., 1997). LaPC4 cells were chosen because they contain wild-type AR and, unlike LNCaP cells that contain a mutated receptor (T877A), do not exhibit altered ligand specificity. From a microarray analysis performed in these cells, we chose 24 androgen-regulated genes (5 androgen-repressed and 19 androgen-activated genes) to generate an AR gene signature. For this study, we selected 25 ligands, several from each cluster, and analyzed their activity on the expression of the selected AR target genes in LaPC4 cells (Figure 4A). The cluster of origin of each compound is designated by color as detailed above and in the legend. Although not identical, the gene expression profile induced by each of the compounds is highly reflective of the results observed in the protein interaction assay. This is best exemplified in Figure 4B where the first principal component for each individual ligand derived from the protein interaction profile is plotted against the first principal component of the transcriptional dataset. The first principal components from both datasets are highly correlative (R^2 = 0.80), suggesting that the specific impact of ligands on the presentation of different protein-protein interaction surfaces is predictive of AR transcriptional activity on endogenous target genes. Of note was the observation that, in LaPC4 cells, the clustering of compounds was equally predictive of target gene repression as it was for target gene activation. These important results suggest that the surfaces on AR involved in transcriptional activation might also be involved in transcriptional repression.

We next tested whether the linear correlation between the protein-protein interaction profiles and transcriptional activity was also reflected in complex androgen actions (e.g., cell proliferation). Thus, the ability of selected reference AR ligands (20 ligands total with representatives from each cluster) to stimulate LaPC4 cell proliferation was assessed (Figure 5). As can be seen, ligands in clusters 1 to 6 stimulate little to no LaPC4 proliferation. However, with the exception of GW980 in cluster 8, all compounds in cluster 7 and higher stimulate LaPC4 proliferation with approximately the same efficacy. Interestingly, unlike transcriptional activity, proliferation does not appear to be a graded response. Instead, the proliferative capacity of the compounds exhibits a threshold effect with the transition point lying between clusters 6 and 7. The significance of this finding is currently being evaluated.

DISCUSSION

It is now generally accepted that the overall conformation of many NRs is determined by the nature of the bound ligand and that it is the ability of the cells to distinguish between differently conformed receptors that dictates pharmacological response. Central to this hypothesis is the idea that changes in conformation result in the presentation of different protein-protein interaction surfaces on the receptor and the subsequent recruitment of functionally distinct cofactors. Indeed, it has been shown that the relative agonist/antagonist activity of SERMs like tamoxifen and

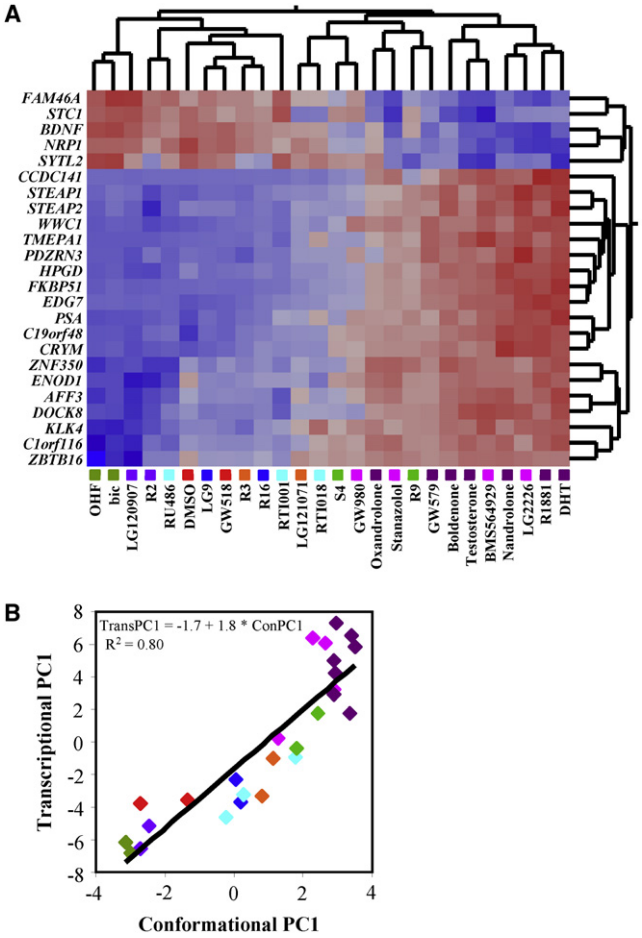


Figure 4. AR Ligand-Induced Conformation Correlates with Transcriptional Activity

(A) Dendogram showing relationship of SARMs based on transcriptional activity of AR gene signature in LaPC4 cells. Gene expression profiles of 19 genes induced and 5 genes repressed by R1881 treatment were generated using 25 AR ligands and vehicle (DMSO). The profiles were analyzed with the Ward hierarchical cluster algorithm using standardized data. The conformation-based cluster of each SARM is represented by color as depicted in Figure 2.

(B) Correlation between endogenous gene transcriptional activity and ligand-induced AR conformation. Principle component analysis was performed on the transcriptional data presented in (A) and principle component 1 was plotted versus the first principle component derived from the conformational profile (Figure 2).

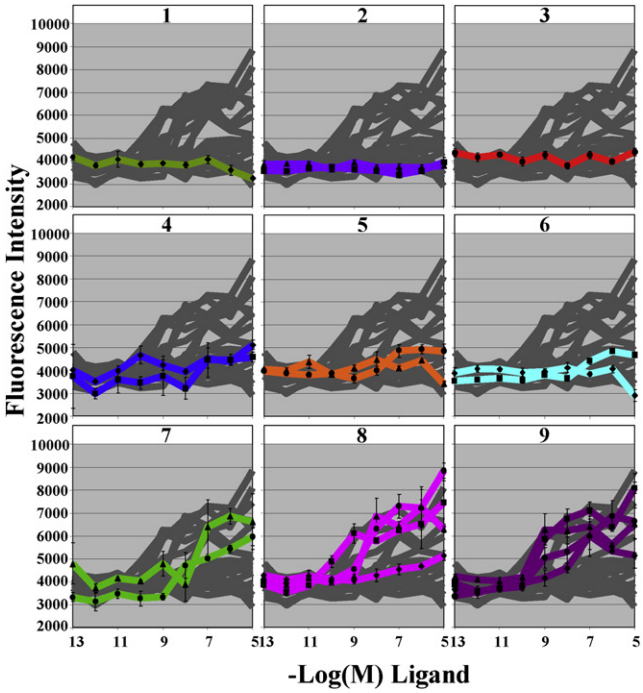


Figure 5. Ligand-Induced AR Conformation Is Predictive of Prostate Cancer Cell Proliferation

LaPC4 cells were seeded for 3 days in medium containing charcoal stripped serum. On days 3, 6, and 9 the cells were treated with the indicated concentrations of AR ligand. Each panel represents compound(s) from the conformational-based compound clusters (1, OHF; 2, ■ – LG 120907, ▲ – R2; 3, GW518; 4, ◆ – LG9, ■ – R16; 5 ▲ – R3, ● – LG121071; 6, ◆ – RU486, ■ – RTI018; 7, ▲ – S4, ● – R9; 8 ◆ – GW980, ■ – LG2226, ▲ – BMS564929, ● – stanozolol; 9 ◆ – GW579, ■ - testosterone, ▲ – DHT, ● – R1881). Proliferation was determined by measuring total cellular DNA content on day 10. The data presented are representative of three independent experiments. The error bars represent the standard deviation of a single experiment performed in triplicate.

raloxifene can be regulated by manipulating the expression of selected coactivators and corepressors in cells (Keeton and Brown, 2005; Shang and Brown, 2002). However, the extent to which differential protein-protein interactions can be used in a prospective manner to predict the pharmacological actions of NR ligands has not been evaluated. In this study, using AR as a model, we have shown that the presentation of protein-protein interaction surfaces on this receptor can be regulated by different ligands and that the resultant profiles are highly predictive of the biological activity of the receptor. Interestingly, we did not find any proteins that interact exclusively with one particular AR-ligand complex. Instead, our studies suggest that it is the relative, rather than absolute, binding of interacting proteins to AR that determines the pharmacological response of different AR modulators.

One of the interesting observations gleaned from the studies presented here is that the protein-protein interaction surfaces on AR presented upon binding the nonsteroidal SARMs, S4 (cluster 7), BMS564929 (cluster 8), and LG2226 (cluster 8), are similar to those defined by the canonical agonists, DHT and T (cluster 9). These findings, using full-length AR, are consistent with recent crystallographic studies that showed that the structure of the AR-LBD in the presence of either a SARM or a full agonist were nearly identical (Ostrowski et al., 2007; Wang et al., 2006). These results are in stark contrast to what has been observed with ER, where SERMs have been shown to induce a conformation in ER that is quite distinct from classical agonists (Brzozowski et al., 1997; Shiau et al., 1998; Wu et al., 2005). This suggests that although SARMs and SERMs are similar in that they are both capable of mediating the tissue-specific agonist activities of their respective receptors, they differ in the way they achieve this response. SERMs can best be described as ER antagonists that display partial agonist activities in some ER-responsive tissues like the uterus and bone

(McDonnell et al., 2002), whereas SARMs can best be described as strong AR partial agonists whose level of activity is not sufficient to evoke a proliferative response in the prostate. Future studies will be aimed at examining compounds in clusters with more antagonist biocharacter (clusters 4–6) in vivo to determine the extent to which we can "dial down" the protein-protein interaction profiles associated with agonists and still retain the desired anabolic activity without prostate stimulation. Compounds from these clusters might possess more favorable SARM pharmacological profiles.

Many of the ligands used in the compound-profiling tool are derived from a similar chemical scaffold(s). Interestingly, subtle chemical changes in a single scaffold can lead to a diverse set of molecules, each with a different biocharacter. For example, many of the sentinel compounds utilized in the profiling tool were based on the hydroxyflutamide chemical scaffold (Table S2). These compounds range from full antagonists to partial agonists with SARM-like properties and can be found populating conformational clusters 1 through 9. Thus, from a single chemical scaffold, we can identify and discriminate compounds that demonstrate considerable diversity with respect to their impact on the presentation of protein-protein interactions surfaces, an activity translated into different pharmacological activities. Importantly, we did not detect a strong correlation between the activity of compounds in our profiling assay and their chemical structures. One notable exception is the RU486-derived compounds found primarily populating conformational cluster 6.

Whereas it has been possible to retrospectively define conformational changes in receptor structure that track with specific pharmacological attributes of NR ligands, it has been difficult to use conformation alone as a predictive surrogate for the biological activity of new chemical entities. To circumvent the limitations of both the crystallographic and peptide profiling approaches, we undertook to develop and validate an assay that reported on the differential presentation of multiple protein-protein interaction surfaces on AR upon binding different ligands. Thus, absent information on the physiological relevance of the interacting proteins identified, we feel that the approach taken here and validated in the context of the androgen-signaling pathway, indicates that the impact of ligands on the presentation of different protein-protein interaction surfaces is the primary, predictable determinant of the pharmacological actions of AR ligands.

SIGNIFICANCE

It is now well established that the impact of a given ligand on NR structure and the effect that this has on the recruitment of functionally distinct cofactors are key determinants of NR pharmacology. Given this insight, it was hypothesized that it might be possible to prospectively define the pharmacological activity of a ligand by assessing its impact on receptor structure. Some progress in this regard has been made using crystallography to look specifically at the impact of different ligands on the conformation of the isolated ligand-binding domain of several receptors (Brzozowski et al., 1997; Matias et al., 2000; Shiau et al., 1998; Wu et al., 2005). In addition, we and others have used combinatorial peptide phage display to survey the presentation of protein-protein interaction surfaces, a surrogate for conformation, on ligand-activated NRs (Connor et al., 2001; Norris et al., 1999; Pearce et al., 2004). However, although somewhat successful, these approaches have only been informative with respect to the impact of ligands on the architecture of a single cofactor-binding domain on the receptor, the AF-2 coactivator binding pocket. We report, in this study, on the development and validation of an approach that circumvents the limitations of previous efforts to link ligand induced changes in receptor structure to specific pharmacological responses. Specifically, we selected AR as a model receptor and used T7 phage display to identify over 150 proteins/polypeptides whose ability to interact with full-length receptor was influenced by the nature of the bound ligand. A subset of the proteins identified was subsequently used to develop a "profiling" tool that allowed us to classify ligands according to their ability to engender different AR-protein interactions. Of specific importance was the finding that the pharmacological activity of previously uncharacterized AR ligands could be predicted in a prospective manner based on the protein-protein interactions that they engender. It is anticipated that in a similar manner it will be possible to develop compound-profiling tools for other NRs.

EXPERIMENTAL PROCEDURES

Chemicals and Plasmids

5α-Dihydrotestosterone, boldenone, stanozolol, nandrolone, testosterone, and oxandrolone were purchased from Steraloids (Newport, RI). R1881 was purchased from PerkinElmer (Waltham, MA). Hydroxyflutamide was purchased from Toronto Research Chemicals (Toronto, Canada). All other AR ligands were synthesized at GlaxoSmithKline. 5xGal4Luc3, MMTV-Luc, pcDNA-AR1-660, and VP16-AR507-919 were described previously (Chang et al., 2005; Chang and McDonnell, 2002). For AR mammalian two-hybrid assays, all insert sequences corresponding to AR-interacting clones were subcloned into the Gateway entry vector pENTR2B (Invitrogen, Carlsbad, CA) followed by recombination into the pM mammalian two-hybrid vector (Clontech, Mountain View, CA) that was Gateway enabled (pM-GB). pSG5-AR was a gift from T. Willson (GlaxoSmithKline, Durham, NC). pCMX-Gal4-C'SMRT was a gift from J.D. Chen (University of Medicine and Dentistry of New Jersey, Newark, NJ). pM-ARA54 was generated by polymerase chain reaction (PCR) amplification of ARA54 cDNA (corresponding to aa 361–474), followed by subcloning into the pM vector. phRL-CMV (Renilla-Luc) was purchased from Promega (Madison, WI).

T7 Phage Display

Full-length baculovirus purified AR was used as bait to screen for T7 phage expressing AR-interacting proteins. Additional details can be found in Supplemental Experimental Procedures.

Transfection Assays

HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS, Invitrogen). For transfection, cells were seeded into either 24 or 96 well cell culture plates in DMEM supplemented with charcoal stripped serum (Hyclone, Logan, UT) and were transfected with Lipofectin (Invitrogen) according to the manufacturer's instructions. For AR mammalian two-hybrid assays, the DNA mixture transfected into the cells consisted of VP16-AR, 5XGalLuc3, pM-T7 clone, and pCMV-βGal. For AR N- and C-terminal interaction assays, the DNA mixture consisted of pcDNA-AR1-660, VP16-AR507-919, MMTV-luciferase and pCMV-βGal. For AR transcriptional assays, the DNA mixture consisted of pSG5-AR, MMTV-luciferase, and pCMV-βGal. For AR cofactor profiling assays, the DNA mixture consisted of VP16-AR, 5XGalLuc3, pM-T7 clone, and phRL-CMV. Immediately following transfection, cells were induced with hormone for 48 hr. Cells

were then lysed and firefly luciferase (reporter) and β-galactosidase (transfection normalization) assays were performed. For AR-cofactor profiling assays, Renilla luciferase assay was used as control for transfection efficiency.

Proliferation Assay

LaPC4 cells were maintained in Iscove's DMEM supplemented with 15% FCS plus R1881 (0.1 nM). For proliferation assays, cells were plated in 96 well plates in Iscove's DMEM supplemented with charcoal stripped FCS (15%) at 10,000 cells/well. Following 72 hr incubation, cells were induced with ligand or vehicle treatment. Cells were induced with ligand an additional two times at 72 hr intervals. Twenty-four hours after the final hormone treatment, cells were assayed for DNA content using Hoechst dye (excitation 346 nm, emission 460 nm).

RNA Isolation and Real-Time PCR

LaPC4 cells were treated for 24 hr with ligand and RNA was isolated using the Aurum™ total RNA isolation kit (Bio-Rad, Hercules, CA). RNA (1 μg) was reverse transcribed using the Bio-Rad iScript cDNA synthesis kit. Real-time PCR was performed using the Applied Biosystems (Foster City, CA) 7300 instrument and iQ SYBR Green supermix (Bio-Rad). GAPDH expression was used to normalize all real-time data. Sequences for gene-specific primers can be found in Table S3.

Statistics

For gene ontology analysis, the relative overrepresentation of GO protein modules was established using ONTO-Express (Draghici et al., 2003) and Source (http://source.stanford.edu/cgi-bin/source/sourceSearch). Additional details can be found in Supplemental Experimental Procedures.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, two figures, and three tables and can be found with this article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(09)00044-1.

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REFERENCES

Brzozowski, A.M., Pike, A.C., Dauter, Z., Hubbard, R.E., Bonn, T., Engstrom, O., Ohman, L., Greene, G.L., Gustafsson, J.A., and Carlquist, M. (1997). Molecular basis of agonism and antagonism in the oestrogen receptor. Nature *389*, 753–758.

Cadilla, R., and Turnbull, P. (2006). Selective androgen receptor modulators in drug discovery: medicinal chemistry and therapeutic potential. Curr. Top. Med. Chem. *6*, 245–270.

Chang, C., Norris, J.D., Gron, H., Paige, L.A., Hamilton, P.T., Kenan, D.J., Fowlkes, D., and McDonnell, D.P. (1999). Dissection of the LXXLL nuclear receptor-coactivator interaction motif using combinatorial peptide libraries: discovery of peptide antagonists of estrogen receptors alpha and beta. Mol. Cell. Biol. *19*, 8226–8239.

Chang, C.Y., Abdo, J., Hartney, T., and McDonnell, D.P. (2005). Development of peptide antagonists for the androgen receptor using combinatorial peptide phage display. Mol. Endocrinol. *19*, 2478–2490.

Chang, C.Y., and McDonnell, D.P. (2002). Evaluation of ligand-dependent changes in AR structure using peptide probes. Mol. Endocrinol. *16*, 647–660.

Chang, C.Y., and McDonnell, D.P. (2005). Androgen receptor-cofactor interactions as targets for new drug discovery. Trends Pharmacol. Sci. *26*, 225–228.

Cole, R.C., Flaws, J.A., and Bush, T.L. (1998). Effects of raloxifene in postmenopausal women. N. Engl. J. Med. *338*, 1313–1314.

Connor, C.E., Norris, J.D., Broadwater, G., Willson, T.M., Gottardis, M.M., Dewhirst, M.W., and McDonnell, D.P. (2001). Circumventing tamoxifen resistance in breast cancers using antiestrogens that induce unique conformational changes in the estrogen receptor. Cancer Res. *61*, 2917–2922.

Dalton, J.T., Mukherjee, A., Zhu, Z., Kirkovsky, L., and Miller, D.D. (1998). Discovery of nonsteroidal androgens. Biochem. Biophys. Res. Commun. *244*, 1–4.

Draghici, S., Khatri, P., Bhavsar, P., Shah, A., Krawetz, S.A., and Tainsky, M.A. (2003). Onto-Tools. The toolkit of the modern biologist: Onto-Express, Onto-Compare, Onto-Design and Onto-Translate. Nucleic Acids Res. *31*, 3775–3781.

Gao, W., and Dalton, J.T. (2007). Expanding the therapeutic use of androgens via selective androgen receptor modulators (SARMs). Drug Discov. Today *12*, 241–248.

Hamann, L.G., Higuchi, R.I., Zhi, L., Edwards, J.P., Wang, X.N., Marschke, K.B., Kong, J.W., Farmer, L.J., and Jones, T.K. (1998). Synthesis and biological activity of a novel series of nonsteroidal, peripherally selective androgen receptor antagonists derived from 1,2-dihydropyridono[5,6-g]quinolines. J. Med. Chem. *41*, 623–639.

He, B., Kemppainen, J.A., and Wilson, E.M. (2000). FXXLF and WXXLF sequences mediate the NH2-terminal interaction with the ligand binding domain of the androgen receptor. J. Biol. Chem. *275*, 22986–22994.

He, B., Minges, J.T., Lee, L.W., and Wilson, E.M. (2002). The FXXLF motif mediates androgen receptor-specific interactions with coregulators. J. Biol. Chem. *277*, 10226–10235.

He, B., and Wilson, E.M. (2002). The NH(2)-terminal and carboxyl-terminal interaction in the human androgen receptor. Mol. Genet. Metab. *75*, 293–298.

Heery, D.M., Kalkhoven, E., Hoare, S., and Parker, M.G. (1997). A signature motif in transcriptional co-activators mediates binding to nuclear receptors. Nature *387*, 733–736.

Hsiao, P.W., Lin, D.L., Nakao, R., and Chang, C. (1999). The linkage of Kennedy's neuron disease to ARA24, the first identified androgen receptor polyglutamine region-associated coactivator. J. Biol. Chem. *274*, 20229–20234.

Jung, C., Kim, R.S., Zhang, H.J., Lee, S.J., and Jeng, M.H. (2004). HOXB13 induces growth suppression of prostate cancer cells as a repressor of hormone-activated androgen receptor signaling. Cancer Res. *64*, 9185–9192.

Juzumiene, D., Chang, C.Y., Fan, D., Hartney, T., Norris, J.D., and McDonnell, D.P. (2005). Single-step purification of full-length human androgen receptor. Nucl. Recept. Signal. *3*, e001.

Kallio, P.J., Palvimo, J.J., Mehto, M., and Janne, O.A. (1994). Analysis of androgen receptor-DNA interactions with receptor proteins produced in insect cells. J. Biol. Chem. *269*, 11514–11522.

Kang, H.Y., Yeh, S., Fujimoto, N., and Chang, C. (1999). Cloning and characterization of human prostate coactivator ARA54, a novel protein that associates with the androgen receptor. J. Biol. Chem. *274*, 8570–8576.

Kazmin, D., Prytkova, T., Cook, C.E., Wolfinger, R., Chu, T.M., Beratan, D., Norris, J.D., Chang, C.Y., and McDonnell, D.P. (2006). Linking ligand-induced alterations in androgen receptor structure to differential gene expression: a first step in the rational design of selective androgen receptor modulators. Mol. Endocrinol. *20*, 1201–1217.

Keeton, E.K., and Brown, M. (2005). Cell cycle progression stimulated by tamoxifen-bound estrogen receptor-alpha and promoter-specific effects in breast cancer cells deficient in N-CoR and SMRT. Mol. Endocrinol. *19*, 1543–1554.

Kemppainen, J.A., Langley, E., Wong, C.I., Bobseine, K., Kelce, W.R., and Wilson, E.M. (1999). Distinguishing androgen receptor agonists and antagonists: distinct mechanisms of activation by medroxyprogesterone acetate and dihydrotestosterone. Mol. Endocrinol. *13*, 440–454.

Khatri, P., Bhavsar, P., Bawa, G., and Draghici, S. (2004). ONTO-tools: an ensemble of web-accessible, ontology-based tools for the functional design and interpretation of high-throughput gene expression experiments. Nucleic Acids Res. *32*, W449–W456.

Klein, K.A., Reiter, R.E., Redula, J., Moradi, H., Zhu, X.L., Brothman, A.R., Lamb, D.J., Marcelli, M., Belldegrun, A., Witte, O.N., et al. (1997). Progression of metastatic human prostate cancer to androgen independence in immunodeficient SCID mice. Nat. Med. *3*, 402–408.

Lee, J.W., Choi, H.S., Gyuris, J., Brent, R., and Moore, D.D. (1995). Two classes of proteins dependent on either the presence or absence of thyroid hormone for interaction with the thyroid hormone receptor. Mol. Endocrinol. *9*, 243–254.

Lin, H.K., Hu, Y.C., Lee, D.K., and Chang, C. (2004). Regulation of androgen receptor signaling by PTEN (phosphatase and tensin homolog deleted on chromosome 10) tumor suppressor through distinct mechanisms in prostate cancer cells. Mol. Endocrinol. *18*, 2409–2423.

Love, R.R., Mazess, R.B., Barden, H.S., Epstein, S., Newcomb, P.A., Jordan, V.C., Carbone, P.P., and DeMets, D.L. (1992). Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. N. Engl. J. Med. *326*, 852–856.

Matias, P.M., Donner, P., Coelho, R., Thomaz, M., Peixoto, C., Macedo, S., Otto, N., Joschko, S., Scholz, P., Wegg, A., et al. (2000). Structural evidence for ligand specificity in the binding domain of the human androgen receptor. Implications for pathogenic gene mutations. J. Biol. Chem. *275*, 26164–26171.

McDonnell, D.P., Clemm, D.L., Hermann, T., Goldman, M.E., and Pike, J.W. (1995). Analysis of estrogen receptor function in vitro reveals three distinct classes of antiestrogens. Mol. Endocrinol. *9*, 659–669.

McDonnell, D.P., Wijayaratne, A., Chang, C.Y., and Norris, J.D. (2002). Elucidation of the molecular mechanism of action of selective estrogen receptor modulators. Am. J. Cardiol. *90*, 35F–43F.

McEwan, I.J., and Gustafsson, J. (1997). Interaction of the human androgen receptor transactivation function with the general transcription factor TFIIF. Proc. Natl. Acad. Sci. USA *94*, 8485–8490.

Miner, J.N., Chang, W., Chapman, M.S., Finn, P.D., Hong, M.H., Lopez, F.J., Marschke, K.B., Rosen, J., Schrader, W., Turner, R., et al. (2007). An orally active selective androgen receptor modulator is efficacious on bone, muscle, and sex function with reduced impact on prostate. Endocrinology *148*, 363–373.

Negro-Vilar, A. (1999). Selective androgen receptor modulators (SARMs): a novel approach to androgen therapy for the new millennium. J. Clin. Endocrinol. Metab. *84*, 3459–3462.

Nishimura, K., Ting, H.J., Harada, Y., Tokizane, T., Nonomura, N., Kang, H.Y., Chang, H.C., Yeh, S., Miyamoto, H., Shin, M., et al. (2003). Modulation of androgen receptor transactivation by gelsolin: a newly identified androgen receptor coregulator. Cancer Res. *63*, 4888–4894.

Norris, J.D., Paige, L.A., Christensen, D.J., Chang, C.Y., Huacani, M.R., Fan, D., Hamilton, P.T., Fowlkes, D.M., and McDonnell, D.P. (1999). Peptide antagonists of the human estrogen receptor. Science *285*, 744–746.

Ostrowski, J., Kuhns, J.E., Lupisella, J.A., Manfredi, M.C., Beehler, B.C., Krystek, S.R., Jr., Bi, Y., Sun, C., Seethala, R., Golla, R., et al. (2007). Pharmacological and x-ray structural characterization of a novel selective androgen receptor modulator: potent hyperanabolic stimulation of skeletal muscle with hypostimulation of prostate in rats. Endocrinology *148*, 4–12.

Pearce, K.H., Iannone, M.A., Simmons, C.A., and Gray, J.G. (2004). Discovery of novel nuclear receptor modulating ligands: an integral role for peptide interaction profiling. Drug Discov. Today *9*, 741–751.

Sathya, G., Chang, C.Y., Kazmin, D., Cook, C.E., and McDonnell, D.P. (2003). Pharmacological uncoupling of androgen receptor-mediated prostate cancer cell proliferation and prostate-specific antigen secretion. Cancer Res. *63*, 8029–8036.

Sato, M., Rippy, M.K., and Bryant, H.U. (1996). Raloxifene, tamoxifen, nafoxidine, or estrogen effects on reproductive and nonreproductive tissues in ovariectomized rats. FASEB J. *10*, 905–912.

Shang, Y., and Brown, M. (2002). Molecular determinants for the tissue specificity of SERMs. Science *295*, 2465–2468.

Shiau, A.K., Barstad, D., Loria, P.M., Cheng, L., Kushner, P.J., Agard, D.A., and Greene, G.L. (1998). The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell *95*, 927–937.

Ting, H.J., Yeh, S., Nishimura, K., and Chang, C. (2002). Supervillin associates with androgen receptor and modulates its transcriptional activity. Proc. Natl. Acad. Sci. USA *99*, 661–666.

Tzukerman, M.T., Esty, A., Santiso-Mere, D., Danielian, P., Parker, M.G., Stein, R.B., Pike, J.W., and McDonnell, D.P. (1994). Human estrogen receptor transactivational capacity is determined by both cellular and promoter context and mediated by two functionally distinct intramolecular regions. Mol. Endocrinol. *8*, 21–30.

Vogel, V.G., Costantino, J.P., Wickerham, D.L., Cronin, W.M., Cecchini, R.S., Atkins, J.N., Bevers, T.B., Fehrenbacher, L., Pajon, E.R., Jr., Wade, J.L., 3rd, et al. (2006). Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. JAMA *295*, 2727–2741.

Wang, F., Liu, X.Q., Li, H., Liang, K.N., Miner, J.N., Hong, M., Kallel, E.A., van Oeveren, A., Zhi, L., and Jiang, T. (2006). Structure of the ligand-binding domain (LBD) of human androgen receptor in complex with a selective modulator LGD2226. Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun. *62*, 1067–1071.

Wang, Q., Sharma, D., Ren, Y., and Fondell, J.D. (2002). A coregulatory role for the TRAP-mediator complex in androgen receptor-mediated gene expression. J. Biol. Chem. *277*, 42852–42858.

Ward, J.H. (1963). Hierarchical grouping to optimize an objective function. J. Am. Stat. Assoc. *58*, 236–244.

Wu, Y.L., Yang, X., Ren, Z., McDonnell, D.P., Norris, J.D., Willson, T.M., and Greene, G.L. (2005). Structural basis for an unexpected mode of SERM-mediated ER antagonism. Mol. Cell *18*, 413–424.

Yin, D., Gao, W., Kearbey, J.D., Xu, H., Chung, K., He, Y., Marhefka, C.A., Veverka, K.A., Miller, D.D., and Dalton, J.T. (2003). Pharmacodynamics of selective androgen receptor modulators. J. Pharmacol. Exp. Ther. *304*, 1334–1340.

