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LETTER

A Cellular Conformation-Based Screen for Androgen Receptor Inhibitors

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ABSTRACT The androgen receptor (AR), a member of the steroid nuclear receptor family of transcription factors, regulates a wide range of physiological processes. Androgen signaling is also associated with numerous human diseases, including prostate cancer. All current antiandrogen therapies reduce ligand access to AR, whether by competitive antagonism or inhibition of androgen production, but are limited by acquired resistance and serious side-effects. Thus, novel antiandrogens that target events subsequent to ligand binding could have important therapeutic value. We developed a high throughput assay that exploits fluorescence resonance energy transfer (FRET) to measure ligand-induced conformation change in AR. We directly compared this assay to a transcription-based assay in a screen of FDA-approved compounds and natural products. The FRET-based screen identified compounds with previously unrecognized antiandrogen activities, with equivalent sensitivity and superior specificity compared to a reporter-based screen. This approach can thus improve the identification of small molecule AR inhibitors.

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The androgen receptor (AR) is a member of the nuclear receptor (NR) superfamily, which consists of a large group of ligand-regulated transcription factors (*1*). AR is expressed in many tissues and influences an enormous range of physiologic processes such as cognition, muscle hypertrophy, bone density, and prostate growth (*2*). AR signaling is directly linked to numerous human disorders including benign prostatic hyperplasia, alopecia, and hirsutism. AR also drives prostate carcinoma proliferation, even in the setting of androgen ablation therapies, and is thus the major therapeutic target for this malignancy (*3*). Existing therapies seek to prevent ligand binding to AR, whether by direct competition or by reduction of serum hormone levels with GNRH agonists or 5-α reductase inhibitors. New classes of AR inhibitors are needed and could have broad therapeutic applications.

AR signaling is complex and highly regulated (Figure 1, panel a). Prior to binding its natural ligand, dihydrotestosterone (DHT), AR associates with a complex of cytoplasmic factors and molecular chaperones that maintain it in a high-affinity ligand binding conformation (*4, 5*). Ligand binding induces an intramolecular conformation change in AR that brings the N- and C-termini into close proximity. Using fluorescence resonance energy transfer (FRET) (Figure 1, panel b), we previously determined that this occurs with a $t_{1/2} \sim 3.5$ min in live cells (*6*) but does not occur in cell lysates (Figure 1, panel c), suggesting that conformation change is not protein autonomous but depends on additional cellular factors. Following ligand binding, AR concentrates in the nucleus, where it binds DNA as a homodimer at specific androgen response elements (AREs) to regulate gene expression. Transcriptional control by AR results from complex interactions with positive (coactivator) and negative (corepressor) factors (*7*) (Figure 1, panel a). The receptor is recycled back to the cytoplasm in a highly regulated process that is independent of receptor degradation (*8*).

AR is regulated by cross-talk pathways that may include post-translational modifications such as phosphorylation, sumolation, and acetylation (*7*). For example, HER-2/neu kinase, keratinocyte growth factor, insulin-like growth factor-1, epidermal growth factor, and cytokines such as IL-6 can activate AR and minimize or possibly negate the requirement for ligand (*9–12*). Inhibiting these and other regulatory pathways may provide alternative methods to block AR activity.

Most screening assays to identify AR inhibitors indirectly measure AR activity using reporter genes. These are potentially vulnerable to nonspecific inhibition at multiple steps interposed between the initial activating event (ligand binding) and the final readout (gene activation) (Figure 1, panel a). As an alternative, we focused on AR ligand-induced conformation change as a highly specific, proximal molecular event in AR signaling. We developed a FRET-based assay to monitor AR conformation change in live cells that is amenable to high-throughput screening (HTS) (Figure 1, panel b).

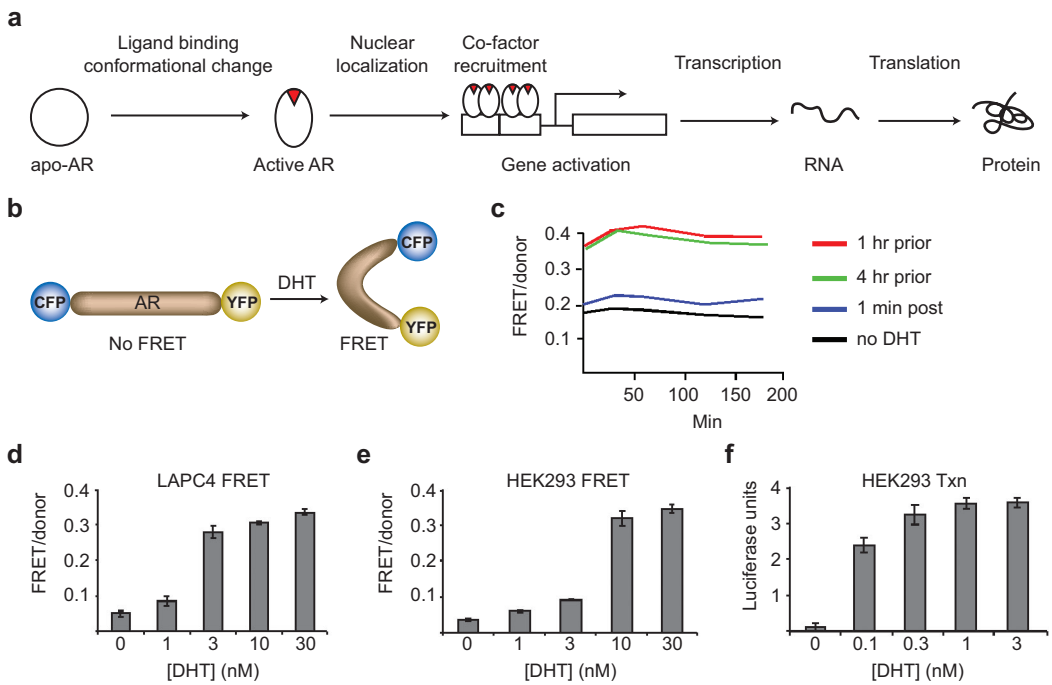

Figure 1. Conformation vs transcription readouts of AR activity. a) Reporter gene assays require multiple steps of cellular activity to produce a signal, beginning with conversion of apo-AR from an inactive to an active state. b) Fusion of cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP) to the N- and C-termini of AR creates a conformational reporter that produces a FRET signal upon hormone activation. c) AR ligand-induced conformational change will not occur in a cell-free extract but is stable if this change is induced prior to cell lysis. HEK293 cells expressing CFP-AR-YFP were treated with 10 nM DHT 1 h or 4 h prior to lysis, immediately after lysis, or were left untreated. Prior treatment of cells with DHT produced a stable FRET signal after lysis, indicating that AR assumed an active conformation. Treatment after lysis does not trigger the same conformational change, indicating the requirement for an intact cell. d–f) LAPC4/C-AR-Y cells, HEK293/C-AR-Y cells, or HEK293 cells transiently transfected with AR and MMTV-luciferase were evaluated with a dose–response to DHT. Each cell line exhibited a characteristic DHT response, whether by FRET or luciferase activity.

In previous work, we have shown that the fusion of full length AR to cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), termed C-AR-Y, maintains its basic transcriptional activity (*6*). C-AR-Y can be used to measure ligand-induced intramolecular conformation change in real time: in single cells by microscopy or in cell monolayers using a fluorescence plate reader (FPR) (*6*). Here we have compared this FRET-based assay to a transcription-based system in an attempt to identify new classes of AR inhibitors. We have carried out a screen of FDA-approved drugs and natural products and have identified compounds with previously unidentified antiandrogen activities.

RESULTS AND DISCUSSION

Creation and Characterization of FRET and Transcription-Based Reporters. C-AR-Y was stably expressed in HEK293 cells (HEK293/C-AR-Y), a human kidney cell line that does not express AR, and LAPC4 cells (LAPC4/C-AR-Y), an androgen-dependent prostate cancer cell line with endogenous AR expression (*13*). The stable cell lines exhibited DHT-induced FRET signal with a characteristic dose–response (Figure 1, panels d and e). The calculated *Z* values for these stable cell lines in the FRET assay were 0.6 (LAPC4) and 0.5 (HEK293) (*14*). For the transcriptional reporter system, HEK293 cells were transfected with vectors expressing full-length human AR, an androgen-responsive firefly luciferase (MMTV-luc), and an androgen-insensitive SV40 promoter-driven renilla luciferase (pRL-SV40). These cells also exhibited a charac-

TABLE 1. Screening strategy and distribution of hits through the screening process[a]

Screening step		Compounds scoring positive		
		LAPC4 FRET	HEK293 FRET	HEK293 transcription
I	Toxic compounds eliminated	996	898	985
II	Dose–response in primary assay (top 50)	14	18	17
III	AR transcription in LAPC4 cells	13	14	10

[a]C-AR-Y stable cells or transfected transcription reporter cells were cultured in duplicate in the presence of 10 nM DHT and 10 μM of library compounds. Cytotoxic compounds and compounds without consistent activity in duplicate trials were eliminated (**I**). The top 50 hits from each assay were evaluated in detail with a dose–response study (**II**). Compounds that displayed a classic dose–response were considered "validated" hits in the primary assay. This constituted 28–36% of the hits from the primary screen. The validated hits were then tested for efficacy in a secondary assay of endogenous AR transcriptional activity (**III**). The number of compounds validated by effects on endogenous AR transcription in LAPC4 cells is indicated.

teristic dose–response (Figure 1, panel f) with a *Z* value of 0.6, comparable to the FRET assays.

Screening for Novel AR Antagonists: Conformation versus Transcription. Since the FRET assays specifically monitor AR conformational change, we predicted that they might detect novel antiandrogens with a greater degree of specificity than a traditional transcription reporter assay. Consequently, the assays were compared in a screen of the NINDS compound collection (www.msdiscovery.com) (*15, 16*), which consists of 1040 FDA-approved drugs and natural products. This library was chosen for its small size, the potential to rapidly introduce hits into the clinic and because many of the compounds have previously annotated functions, which can facilitate the identification of cellular targets.

Cells were treated with 10 nM DHT for 24 h in the presence of library compounds. Each compound was tested in duplicate on separate plates at 10 μM. Control wells on each plate included no DHT (baseline signal) and DHT without library compound (maximal signal). Hydroxy-flutamide (OH-F, 1 μM), a competitive antagonist of DHT known to inhibit the AR N–C interaction (*6, 17*), was used as a positive control. After 24 h, normalized luciferase or fluorescence signals were measured.

We established a basic algorithm to filter the data and validate compounds from each of the primary screens (Table 1). To be selected for further analysis, a compound had to function in both replicates of the primary screen, each reducing the signal by at least 1 standard deviation from the mean of the maximal DHT-induced signal. This eliminated compounds with a strong effect in only one replicate. Compounds with toxic effects were eliminated based on loss of the raw fluorescence signals in the FRET assays and loss of renilla luciferase activity in the transcription assay. Approximately 5–10% of the library compounds were eliminated due to toxicity in one of the assays (Table 1). Since a goal of this screen was to identify new classes of AR inhibitors, all known competitive antagonists, including all steroidal compounds, were dismissed from further analysis. Compounds that passed these filtering requirements were ranked on the basis of antiandrogen activity.

Each assay effectively sorted compounds according to efficacy (Figure 2, panel a). All three systems were equally sensitive to competitive antagonists, each identifying three of the four known AR antagonists within the library. However, the FRET assay appeared to be more stringent than the transcription-based assay. In HEK293/C-AR-Y cells, 95% of the compounds were eliminated at 4 standard deviations (SD) from the mean maximal signal. In LAPC4/C-AR-Y cells, the same stringency was achieved at 3 SD. Similar stringency was not obtained until 6 SD for the reporter transcription assay (Figure 2, panel a). This implies that the conformational assay is less sensitive to nonspecific cellular perturbation than the reporter transcription assay. The FRET-based assay should thus improve compound detection in larger screens, with a higher specificity and similar sensitivity.

The top 50 hits (~5%) in each assay were re-examined using a dose-titration to validate their activities. Approximately 30–40% of the top 50 hits demonstrated a dose response in each primary assay

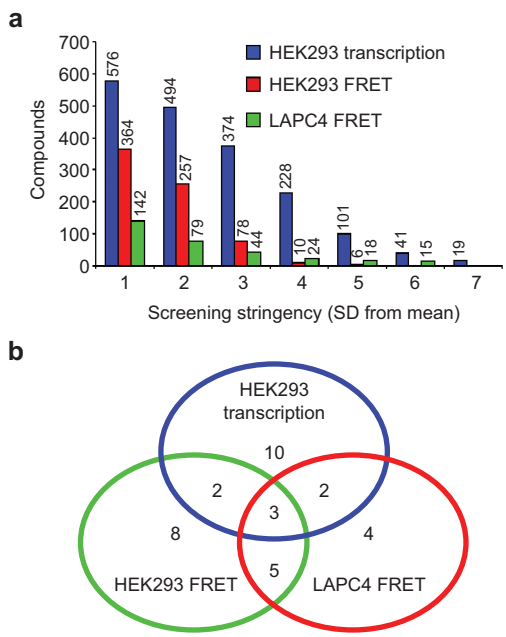

Figure 2. Comparison of stringency and overlap of different assays. a) Hits from the primary screens were compared in terms of reduction of signal by standard deviation (SD) from positive control cells treated with DHT alone. The number of compounds remaining at various stringencies (SD below the mean) was tabulated. The HEK293 transcription assay was the most sensitive to nonspecific inhibition by test compounds, as cutoff stringencies of 5–6 SD below the mean were required to sort the top 5% of hits. HEK293/C-AR-Y cells sorted the top 5% of hits at 3–4 SD below the mean. LAPC4/C-AR-Y cells were the least sensitive to nonspecific inhibition by test compounds, with the top 5% of hits identified at 2–3 SD below the mean. b) The validated hits from each assay were compared in the other assays. Three compounds were active in all three assays.

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TABLE 2. List of hits validated *vs* endogenous AR[a]

		Identified in primary screen		
Compound (related compounds grouped)	**Annotated function**	**LAPC4 FRET**	**HEK293 FRET**	**HEK293 Transcription**
Pyrvinium pamoate	Antihelminthic	X	X	X
Thiabendazole	Antihelminthic		X	
Harmol HCl (β-carboline)	BDZ receptor inverse agonist	X		
Harmaline (β-carboline)	BDZ receptor inverse agonist	X		
Clozapine	GABA receptor antagonist		X	
Clonazepam	GABA receptor antagonist		X	
Esculin (coumarin)	Anticoagulant, vitamin K_1 epoxide inhibitor	X		X
Warfarin (coumarin)	Anticoagulant, vitamin K_1 epoxide inhibitor			X
Peucedanin (coumarin)	Anticoagulant, vitamin K_1 epoxide inhibitor	X		
Scopoletin (coumarin)	Anticoagulant, vitamin K_1 epoxide inhibitor	X	X	
Sulfaquinoxaline	Vitamin K_1 epoxide inhibitor	X		
Emetine	Protein synthesis inhibitor	X		
Melatonin	Central nervous system depressant		X	
Xylazine	Adrenergic receptor antagonists			X
Phenoxybenzamine HCl	Adrenergic receptor antagonists	X		
Mitomycin C	Antibiotic, Antineoplastic			X
Bleomycin	Antibiotic, Antineoplastic			X
Teniposide	Antibiotic, Antineoplastic			X
Oxyquinoline	Antiseptic			X
Pomiferin	Flavonoid			X
Gedunin	HSP90 inhibitor			X
Parthenolide	Nonsteroidal anti-inflammatory			X
Fenofibrate	Antilipemic	X		
Probucol	Antilipemic	X		
Triacetin	Triglyceral antifungal		X	
Exalamide	Antifungal		X	
Memantine HCl	Dopamine agonist	X		
Apomorphine HCl	Dopamine agonist			X
Aminopyridine	Potassium channel blocker	X		
Acetyl tryptophan	Protease inhibitor		X	
Diffratic acid	Unknown		X	
Zoxazolamine	Muscle relaxant		X	
Dioxybenzone	Unknown		X	
Pimethixene maleate	Unknown			X

[a]Compounds identified in secondary analyses as having activity against endogenous AR in an LAPC4 transcription assay were ranked according to efficacy. Annotated functions were gathered from the literature. The rightmost columns indicate which primary screening methodology initially identified the compounds.

(Table 1). To determine the efficacy of each in a secondary assay of endogenous AR activity, the compounds were evaluated in LAPC4 cells transfected with MMTV-luc/pRL-SV40. Most, but not all, of the compounds that were validated in the primary assays were effective in this model of endogenous AR activity (Table 1). The FRET assays were more predictive of efficacy in this secondary model than the transcription assay. The most efficacious compounds, pyrvinium pamoate (PP) ($EC_{50} \sim 12$ nM)

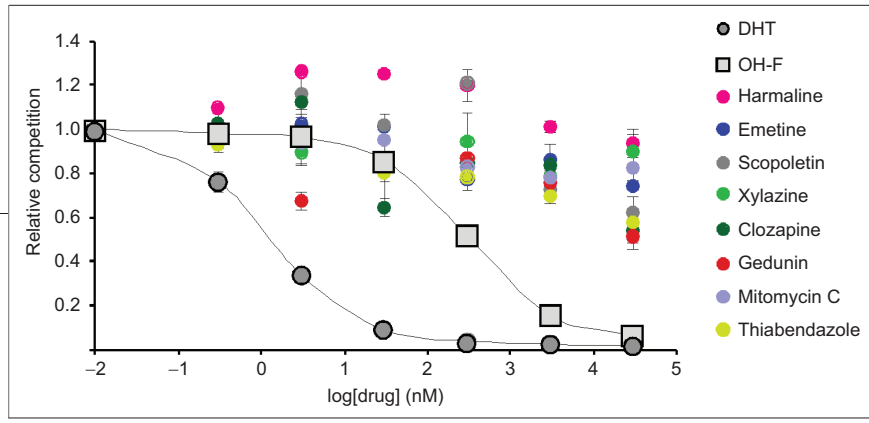

Figure 3. Validated compounds are noncompetitive AR inhibitors. HEK293/C-AR-Y cells were incubated with 3nM [^{3}H] DHT and the indicated compounds. The ability of each compound to compete for binding is expressed relative to the no competition value, which was set to 1. Hydroxyflutamide (OH-F), a competitive antagonist, and unlabeled DHT effectively competed with [^{3}H] DHT for binding. None of the eight AR inhibitors competed for ligand binding.

and harmol hydrochloride (HH) (EC_{50} ~ 106 nM), had higher potency than classical competitive antagonists OH-F (EC_{50} ~ 130 nM) and bicalutamide (BiC) (EC_{50} ~ 1600 nM). Four of the five most efficacious compounds were discovered using the FRET assay; the fifth was a top hit in all three screens (Table 2). Inspection of the primary data revealed that the four compounds identified in the FRET assay also scored positive in the HEK293 transcription assay but were not among the top 50 hits, demonstrating that the noise from such an assay can hinder the detection of truly effective antiandrogens and that use of the FRET assay of conformation change may improve the detection of antiandrogens.

When validated compounds from each assay were subsequently examined for activity in the other two assays, 55–82% of compounds scored positive in at least one other assay (Figure 2, panel b). Three validated compounds scored positive in all three assays, though two of them had not appeared in the top 50 hits of all three primary screens. Interestingly, not all compounds were effective in both FRET assays, implying cell-specific targets that influence AR conformation change.

Noncompetitive AR Inhibitors. No validated hits have structures similar to known AR ligands or inhibitors, suggesting that none would compete for ligand binding. We tested this idea by evaluating a representative sample of validated hits using a whole-cell radioligand competition assay. HEK293/C-AR-Y cells were incubated with [^{3}H] DHT and cold competitors (Figure 3). Unlabeled DHT and the competitive antagonist hydroxyflutamide (OH-F) effectively competed with [^{3}H] DHT for binding. No test compounds examined effectively competed for ligand binding, even at 3–100× the concentration necessary to fully inhibit AR activity. While we cannot rule out a competitive mechanism for all validated hits, our data suggest that most hits from this screen function by a noncompetitive mechanism to inhibit AR activity.

Potential Mechanisms. Coumarin derivatives, including esculin, peucedanin, scopoletin, and warfarin, all inhibited AR activity. Coumarins are widely prescribed anticoagulants that inhibit vitamin K_1 epoxide to interfere with the clotting pathway (*18*). Interestingly, a number of coumarins have been previously determined to have activity against prostate cancer. Decursin induced cell-cycle arrest in prostate cancer derived cells (*19*), and coumarin itself produced tumor regression in clinical trials of metastatic prostate cancer (*20*). Sulfaquinoxaline, another compound identified in this study, is an antimicrobial that also has activity against vitamin K_1 epoxide (*21*). The connection between vitamin K_1 epoxide and AR activity is not immediately obvious but warrants further investigation, as the structure–activity relationship of these compounds suggest a novel mechanism to inhibit AR activity.

A number of inhibitors of γ-aminobutyric acid (GABA)/benzodiazepine (BDZ) receptors also inhibited AR conformation change and transcriptional activity. Two of the most effective AR inhibitors identified in this study, the β-carbolines harmol and harmaline, are well-documented inverse agonists of BDZ receptors (*22, 23*). Clonazepam, a $GABA_A$ selective antagonist (*24*), also scored positive, further implicating the GABA/BDZ receptors in the regulation of AR. It has also been shown that peripheral BDZ receptors (PBR), which are involved in steroid biosynthesis, are not expressed in normal human prostate but are expressed specifically in hyperplastic prostate cells, implicating these receptors in the uncontrolled growth of prostate tissue (*25*). Understanding the relationship between AR and BDZ receptors could lead to novel inhibitory approaches for AR.

Several compounds known to influence the activity of adrenergic receptors (AdRs) were also identified in the screens. Phenoxybenzamine, a nonselective but irreversible inhibitor of α-AdR (*26*), and xylazine, an α-AdR agonist (*27*), both had mild antiandrogen activities. AdRs are highly expressed in prostate tissue. Notably, α-AdR antagonists are used to treat benign prostatic hyperplasia (*28, 29*) and have been proposed as treatments for prostate cancer (*30, 31*). A connection between AdRs and AR may involve signaling through the G-α_s subunit of a G-protein coupled receptor (GPCR) and protein kinase A, using cAMP as an intermediate (*32*). Cross-talk between AR and AdR signaling pathways thus may have important therapeutic implications.

Drug Discovery. The robust nature of the FRET-based assay suggests that it could be used to screen larger libraries. The conformation-based approach can likely be adapted also to identify modulators of other nuclear receptors, including the estrogen receptor (*33*). Further, the ability of microscope-based systems to measure AR conformational change and dimerization with subcellular resolution could make possible the identification of highly specific regulators of receptor function. Additionally, the application of fluorescence readouts of AR conformation in live cells could

be exploited to identify genetic modifiers of receptor function at a single-cell level via flow cytometry. This work validates the concept that conformation-based screening will yield novel AR inhibitors that might otherwise be missed by conventional transcription-based approaches.

Conclusion. Cell-based assays have manifest utility to study nuclear receptors. This work illustrates how a conformational change too complicated to reproduce *in vitro* may be exploited to discover novel inhibitors, many of which may function by mechanisms distinct from competitive antagonists. The ability to identify such "orthogonal" inhibitors is greatly improved by the use of cell-based assays, which permit inhibitors of nonreceptor factors and provide information that cannot be obtained with simple ligand-binding studies (*34*). Indeed, this general approach was recently validated in another study based on an androgen-deprived gene expression profile, which identified inhibitors of hsp90, in addition to three compounds also identified in our screen (*35*). Multiple such orthogonal regulators of AR activity may exist and should facilitate discovery of cellular pathways that control this process. Last, the inhibition of cross-talk pathways that modulate AR signaling in a synergistic fashion may allow significant dose reductions to reduce toxicity for treatments of AR-associated disease.

MATERIALS AND METHODS

Cell Culture. HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with antibiotics and 5% fetal bovine serum (FBS). LAPC4 cells were maintained in phenol-red free RPMI 1640 media supplemented with antibiotics and 10% FBS. After transfection using Lipofectamine (Invitrogen) reagents, 293/C-AR-Y and LAPC4/C-AR-Y cell lines were isolated from single colonies formed under hygromycin selection. Cells were transferred to media containing 5% charcoal-stripped FBS 48 h prior to FRET or transcription assays. The NINDS collection was purchased from Microsource in 96 well plates in DMSO. Pyrvinium pamoate was purchased from MP biomedicines, and all other compounds were purchased from Sigma.

Transcription Assays. Pools of cells were transfected using Lipofectamine Plus reagents (Invitrogen) with plasmids containing full-length AR, MMTV-luciferase and pRL-SV40 (Promega). The following day, the cells were trypsinized and transferred to 96 well plates along with 10 nM DHT and library compounds at 10 μM with a BioMek FX liquid handling robot (Beckman-Coulter). Twenty four hours later, luciferase activity was measured using the Dual luciferase assay kit (Promega). Mean-effect plots (log[compound] vs log[fractional effect]) were generated to determine the EC_{50} values for each compound. Microsoft Excel was used to calculate the statistics for a line using the "least squares" method. The F statistic was used to determine whether the observed relationship between the dependent and independent variables occurred by chance. Only data with an r^2 value greater than 0.95 and an *F* value that was greater than that indicated by the *F* table for $\alpha = 0.05$ were used for analysis. The EC_{50} values are derived from four independent experiments.

FRET Assays. FRET assays were performed as described previously (*36*). Briefly, cells stably expressing C-AR-Y were transferred to black, clear-bottomed 96 well plates along with DHT and library compounds. The cells were fixed in 4% paraformaldehyde and read in PBS on a monochromator-based fluorescence plate reader (Safire, Tecan, Inc.). Each plate contained untransfected, positive, and negative controls. FRET:donor ratios were calculated following background subtraction and correction for acceptor (YFP) contribution to the FRET signal.

Radioligand Competition Binding Assay. HEK293/C-AR-Y cells were seeded in 24-well plates in phenol-red free media containing 5% charcoal-stripped FBS. After 3 days, media were replaced with serum-free media containing 3 nM [^{3}H] DHT in the absence or presence of 0.1−1000-fold molar excess of unlabeled competitor ligands for 90 min at 37 °C. Cells were washed with phosphate buffer, and bound ligand was extracted in ethanol for 30 min at RT and detected using a scintillation counter.

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