Assays to Interrogate the Ability of Compounds to Inhibit the AF-2 or AF-1 Transactivation Domains of the Androgen Receptor

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ABSTRACT

Prostate cancer is the leading cause of cancer and second leading cause of cancer-related death in men in the United States. Twenty percent of patients receiving the standard of care androgen deprivation therapy (ADT) eventually progress to metastatic and incurable castration-resistant prostate cancer (CRPC). Current FDA-approved drugs for CRPC target androgen receptor (AR) binding or androgen production, but only provide a 2- to 5-month survival benefit due to the emergence of resistance. Overexpression of AR coactivators and the emergence of AR splice variants, both promote continued transcriptional activation under androgen-depleted conditions and represent drug resistance mechanisms that contribute to CRPC progression. The AR contains two transactivation domains, activation function 2 (AF-2) and activation function 1 (AF-1), which serve as binding surfaces for coactivators involved in the transcriptional activation of AR target genes. Full-length AR contains both AF-2 and AF-1 surfaces, whereas AR splice variants only have an AF-1 surface. We have recently prosecuted a high-content screening campaign to identify hit compounds that can inhibit or disrupt the protein-protein interactions (PPIs) between AR and transcriptional intermediary factor 2 (TIF2), one of the coactivators implicated in CRPC disease progression. Since an ideal inhibitor/disruptor of AR-coactivator PPIs would target both the AF-2 and AF-1 surfaces, we describe here the development and validation of five AF-2- and three AF-1-focused assays to interrogate and prioritize hits that disrupt both transactivation surfaces. The assays were validated using a test set of seven known AR modulator compounds, including three AR antagonists and one androgen

synthesis inhibitor that are FDA-approved ADTs, two investigational molecules that target the N-terminal domain of AR, and an inhibitor of the Hsp90 (heat shock protein) molecular chaperone.

Keywords: androgen receptor activation function domains, castration-resistant prostate cancer, mammalian 2-hybrid assays

INTRODUCTION

Prostate cancer (PCa) is the second leading type of cancer in men in the United States.¹ Twenty percent of PCa patients develop a terminal form of the disease termed castration-resistant prostate cancer (CRPC) that results in nearly 30,000 deaths per annum in the United States.^{1,2} The mean survival for patients diagnosed with metastatic CRPC is only 9–13 months.² The standard of care treatment options for patients with PCa are surgery and radiation for more localized tumors, and androgen deprivation therapy (ADT) for recurrent or metastatic tumors.^{3,4} Since CRPC cells continue to rely on androgen receptor (AR) signaling for growth and proliferation, ADT drugs modulate AR functions either directly by antagonizing androgen binding to the receptor or indirectly by blocking androgen synthesis.⁵ Unfortunately, ADT drugs also produce adverse events such as osteoporosis, loss of muscle, and muscle weakness. Furthermore, disease flare-up associated with ADT can produce bone pain in patients whose tumors have metastasized to bone tissue, and bursts of tumor growth can also lead to spinal cord compression, or even death.⁶ Around 80% of patients receiving ADT progress to CRPC.⁷ The AR antagonist enzalutamide and the androgen synthesis inhibitor abiraterone, the two drugs most recently approved by the FDA for CRPC, provide only a modest 3- to 5-month patient survival benefit,^{8,9} underscoring the urgent need to identify and develop novel drugs for CRPC treatment. Multiple mechanisms of resistance arise in CRPC cells to render ADT drugs ineffective.^{10–12} AR-dependent resistance mechanisms include AR amplification, intratumoral androgen synthesis, AR mutations that lead to receptor promiscuity, and the emergence of AR splice variants

such as androgen receptor splice variant 7 (AR-V7), which have lost the ligand-binding domain (AR-LBD) in the C-terminus of the receptor and therefore do not respond to the current antiandrogens.^{10–12} Furthermore, overexpression of AR coactivators also contributes to poor CRPC patient outcomes by increasing AR transcriptional activity under androgen-depleted conditions.¹³ Coactivator interactions with both the full-length AR (ARFL) and splice variants like AR-V7 compensate for low cellular dihydrotestosterone (DHT) levels after ADT treatment and result in persistent transcriptional activity.^{13–16}

Under normal androgen-dependent conditions, AR transcriptional activation begins after ligand-activated AR traffics from the cytoplasm into the nucleus, binds to DNA at specific androgen response elements (AREs) on the promoters of AR target genes, and recruits coactivators that enable histone remodeling, additional coactivator recruitment, and subsequent assembly of the transcriptional machinery.¹⁷ AR target genes associated with cellular biosynthesis, survival, and proliferation contribute to PCa disease progression into CRPC.¹⁸ Small molecule drugs that could inhibit AR transcriptional activity represent an alternate mechanism to existing ADTs for the treatment of CRPC. One strategy to inhibit AR-mediated transcriptional activity would be to find small molecules that prevent or disrupt the protein–protein interactions (PPIs) between AR and its coactivators, and thereby block transcription at target gene AREs.^{13,16,19–21} Elevated expression of several AR coactivators have been observed in relapsed PCa patients, including transcriptional intermediary factor 2 (TIF2), steroid receptor coactivator (SRC)-1, RAC3, p300, CBP, Tip60, and ARA 70.^{13,16,19–21} TIF2 (TIF2/SRC-2/NCoA-2/GRIP1) represents a promising drug target for CRPC because TIF2 overexpression occurs in more advanced stages of PCa and increased TIF2 levels correlate with biochemical recurrence in PCa patients.¹³ Small interfering RNA (siRNA) knockdown of TIF2 reduced AR target gene expression and slowed the proliferation of androgen-dependent and androgen-independent PCa cells.¹⁹ The cumulative evidence implicating TIF2 in the progression of PCa into CRPC prompted the development and implementation of a high-content screening (HCS) PPI biosensor (PPIB) assay to identify small molecules that could inhibit the formation of agonist-induced AR-TIF2 PPIs or that could disrupt pre-existing AR-TIF2 complexes.^{22–25}

The AR-TIF2 PPIB assay used two adenovirus constructs to coexpress AR-LBD residues 662–919 as a chimera with red fluorescent protein (AR-RFP), and TIF2 residues 725–840 as a chimera with green fluorescent protein (TIF2-GFP).^{22–25} In cells infected with only the AR-RFP biosensor, which also

contains nuclear import and export sequences, AR-RFP expression is localized predominantly to the cytoplasm, but shifts to the nucleus after exposure to DHT (*Supplementary Fig. S1*). The TIF2 adenovirus contains the receptor interacting domain box III LXXLL (coactivator leucine rich binding motifs) motif that mediates binding to ligand-activated AR-LBD and high-affinity nuclear localization sequence (NLS), and nucleolar localization sequence (NoLS) derived from HIV Rev, which tethers the TIF2-GFP biosensor in the nucleolus of the cell.^{22–25} In cells infected with the TIF2-GFP biosensor alone, TIF2-GFP expression is restricted to the nucleolus within the nucleus irrespective of DHT exposure (*Supplementary Fig. S1*). In untreated cells coinfecting with both AR-RFP and TIF2-GFP biosensors, TIF2-GFP expression is localized only in the nucleoli and AR-RFP expression is predominantly cytoplasmic, but upon exposure to DHT, the AR-RFP biosensor traffics into the nucleus where the PPIs between the AR and TIF2 components result in their colocalization within the nucleoli, as indicated by the bright yellow nucleolar puncta of the FITC and Texas Red composite images (*Supplementary Fig. S1*).^{22–25} Three libraries totaling 143,000 compounds were screened in an AR-TIF2 PPIB HCS campaign to identify concentration-dependent inhibitors and/or disruptors of AR-TIF2 PPIs.²⁵ The AR-TIF2 PPI hits were then triaged in counter screens designed to identify and eliminate compounds that interfered with the PPIB assay format, non-specifically blocked nuclear receptor (NR) trafficking to the nucleus, or reduced AR expression and/or restricted its localization to the cytoplasm.^{26–32}

The AR has two major transactivation domains, activation function 2 (AF-2) and activation function 1 (AF-1), which serve as binding surfaces for coactivators that activate AR gene transcription.^{14,15,33–36} The AF-2 binding surface is formed by helix 12 in the C-terminal domain of the receptor after DHT binds to the AR-LBD,^{35–39} and enables the interactions between AR and the cohorts of coactivators that modulate AR transcriptional activity. The AF-2 surface interacts with coactivators containing binding motifs encoded by LXXLL and/or FXXLF sequences.^{14,37–39} The AF-1 binding surface is located in the intrinsically disordered N-terminal domain of AR (AR-NTD) where the transactivation unit 1 (TAU1) and transactivation unit 5 (TAU5) subregions are as important for ligand-independent transactivation.^{14,34,40,41} TAU5 is required for the recruitment of p160 SRCs 1–3, of which TIF2 (SRC2) is a member.^{14,34,40,41} The AR-NTD AF-1 region is transcriptionally active in the absence of androgens, and AR-NTD constructs lacking an AR-LBD activate reporter genes to the same degree as AR-FL in the presence of ligand.^{14,15,34,42,43} The AF-1 surface is particularly important

to the transcriptional activity of AR splice variants, which lack a LBD and AF-2 surface.^{44–46} AF-1 containing AR-V7 splice variants, which are upregulated in CRPC patients who have relapsed on ADT, also require coactivators to activate gene transcription.^{14,15,34,40,42}

Since the design of the AR-TIF2 PPIB assay might be biased toward finding hits that preferentially inhibited or disrupted DHT-induced AR-LBD AF-2 surface binding with TIF2 Box III LXXLL motifs,^{22–25} we developed a panel of AF-2- and AF-1-focused assays to characterize the transactivation domain profiles of our hits and to prioritize those that might be effective against both AR-FL and AR splice variants. AF-2-focused assays include mammalian 2-hybrid (M2H) PPI assays between the AR-LBD and the p160 coactivators TIF2 and SRC-1, a PSA6.1-Luc (luciferase) reporter assay conducted in a PC3 cell line stably expressing AR-FL-GFP, an AR-LBD tritiated dihydrotestosterone (³H-DHT) radio ligand binding assay, and an AR-LBD TIF2 Box III-LXXLL peptide binding assay. The AR-NTD AF-1 surface-focused assays include a constitutive pGAL4 (DNA-binding domain of the Gal4 protein derived from yeast)-AR-NTD transactivation assay and two reporter assays developed in PC3 cells that stably express AR-V7-GFP, a PSA6.1-Luc, and a ubiquitin-conjugating enzyme E2C (UBE2C)-Luc reporter assay. This article describes the development and optimization of the AF-2 and AF-1 assays and their validation using seven known AR modulator compounds, including four FDA-approved ADTs, two investigational molecules that target the AR-NTD, and an inhibitor of the Hsp90 (heat shock protein) molecular chaperone (*Supplementary Fig. S2*).

MATERIALS AND METHODS

Reagents

Formaldehyde, DHT, flutamide, bicalutamide, enzalutamide (MDV3100), EPI-001, abiraterone acetate, 17-AAG (17-allylaminogeldanamycin), and Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) were purchased from Sigma-Aldrich (St. Louis, MO). Compound 10, 2-[(isoxazol-4-ylmethyl)thio]-1-(4-phenylpiperazin-1-yl)ethanone^{47–49} was kindly provided by Dr. Zhou Wang in the Department of Urology of the University of Pittsburgh Hillman Cancer Institute (Pittsburgh, PA), and Hoechst 33342 was purchased from Invitrogen (Carlsbad, CA). Dimethyl sulfoxide (DMSO; 99.9% high-performance liquid chromatography grade, under argon) was from Alfa Aesar (Ward Hill, MA). Dulbecco's Mg²⁺- and Ca²⁺-free phosphate-buffered saline (PBS) were purchased from Corning (Tewksbury, MA). The AlphaScreen Histidine (Nickel Chelate) Detection Kit, 500 assay points was purchased from Perkin Elmer (Waltham, MA), and Geneticin[™] Selective Antibiotic (G418 Sulfate) pow-

der was purchased from Fisher Scientific (Pittsburgh, PA). T4 DNA Ligase, *Bam*HI (R0136S), and *Eco*RI (R3101S) were purchased from New England Biolabs (Ipswich, MA). FuGENE[™] 6 and FuGENE HD transfection Reagents were purchased from Promega (Madison, WI). Phusion High-Fidelity DNA Polymerase (F530S) was purchased from Fisher Scientific. Bright-Glo[™] Luciferase Assay System was purchased from Promega. DHT [1,2,4,5,6,7-³H(N)]-(5 α -ANDROSTAN-17 β -3-ol) was purchased from Perkin Elmer.

Cell Lines and Tissue Culture

PC3 cells were provided by the National Cancer Institute (NCI) as part of the NCI 60 tumor cell line panel. PC3 cells were maintained in RPMI 1640 (Gibco, Gaithersburg, MD) medium with 2 mM L-glutamine (Invitrogen) that was supplemented with 10% fetal bovine serum (FBS; Gemini Bio-Products, West Sacramento, CA), and 100 U/mL penicillin and streptomycin (Invitrogen).

PC3 cells that stably express AR-V7-GFP were kindly provided by Dr. Michael Mancini in the Departments of Molecular and Cellular Biology, and Pharmacology and Chemical Biology, Baylor College of Medicine, Houston, TX. PC3-AR-V7-GFP cells were maintained in Dulbecco's modified Eagle medium: nutrient mixture F-12 (DMEM/F12; Gibco) and supplemented with 10% FBS and 500 μ g/mL geneticin (G418; Fisher Scientific).

We developed a PC3 cell line that stably expresses AR-FL-GFP by transfecting PC3 cells with a pEGFP-C1-AR-FL expression plasmid kindly provided by Dr. Michael Mancini (Baylor College of Medicine). After transfection of PC3 cells with the pEGFP-C1-AR-FL expression plasmid, cells were placed under selection in culture media containing 600 μ g/mL geneticin (G418; Fisher Scientific), and underwent three rounds of cell sorting for EGFP expression based on the EGFP mean fluorescent intensity measured and sorted on a BD FACSaria (BD Biosciences, San Jose, CA) performed by Dr. Hongmei Shen in the University of Pittsburgh Flow Cytometry Core Facility. The final PC3-AR-FL-GFP cell line was maintained in culture in RPMI 1640 (Gibco) media and supplemented with 10% FBS (Gemini Bio-Products) and 600 μ g/mL geneticin (G418; Fisher Scientific).

Human embryonic kidney 293 cells (HEK 293 cells; CRL-1537) were purchased from the American Type Culture Collection (Manassas, VA) and were maintained in DMEM (Cellgro10013CV; Corning) with 2 mM L-glutamine (Invitrogen) that was supplemented with 10% FBS (Gemini Bio-Products), and 100 U/mL penicillin and streptomycin (Invitrogen).

All cell lines were maintained in a humidified incubator at 37°C, 5% CO₂, and 95% humidity.

Compounds and Compound Handling

To determine 50% inhibition concentrations (IC_{50}) in each assay, 10-point twofold serial dilutions of test compounds in 100% DMSO were performed by using a 384-well P30 dispensing head on the Janus MDT automated liquid handling platform (Perkin Elmer). Daughter plates containing 2 μ L of the serially diluted compounds in DMSO were prepared and replicated from the 384-well serial dilution master plates by using the Janus MDT platform that was outfitted with a 384-well transfer head. Aluminum adhesive plate seals were applied, and plates were stored at -20°C . For testing in the bioassays, daughter plates were withdrawn from -20°C storage, thawed to ambient temperature, and centrifuged for 1 min at 100 *g*, and the plate seals were removed before the transfer of 38 μ L of serum-free media (SFM) into wells by using a Matrix pipettor (Thermo Fisher Scientific, Waltham, MA), to generate an intermediate stock concentration of validation compounds ranging from 0.977 to 500 μ M (5.0% DMSO). The diluted compounds were mixed by repeated aspiration and dispensation by using a 384-well P30 dispensing head on the Janus MDT platform and then, 5 μ L of diluted compounds was transferred to the wells of assay plates to provide a final concentration response ranging from 0.0977 to 50 μ M (0.5% DMSO).

AR-TIF2 PPIB Assay

The AR-TIF2 PPIB HCS assay was performed in U-2 osteosarcoma cell line (U-2 OS) cells as described previously.^{22–25} Briefly, U-2 OS cells were coinfecting with recombinant adenovirus biosensor expression constructs and seeded at 2,500 cells per well in 384-well collagen-coated microplates (No. 781956; Greiner Bio-One), and plates were incubated overnight at 37°C in 5% CO_2 and 95% humidity. To determine if compounds could block DHT-induced AR-TIF2 PPI formation, assay plates were preincubated with compounds for 3 h before the addition of 25 nM DHT for 90 min. To investigate whether compounds could disrupt pre-existing AR-TIF2 PPI complexes, assay plates were preincubated with 25 nM DHT for 90 min before the transfer of compounds, and an additional 3-h incubation. Maximum plate control wells ($n=32$) in columns 1 and 2 were exposed to 25 nM DHT and $\leq 0.25\%$ DMSO, and minimum plate control wells ($n=32$) in columns 23 and 24 were treated with $\leq 0.25\%$ DMSO. Diluted compounds, DHT or DMSO (5 μ L), were transferred at the indicated concentrations as described above. After the appropriate incubation time with compounds and controls, assay plates were fixed by the addition of 50 μ L of prewarmed (37°C) 7.4% formaldehyde and 2 μ g/mL Hoechst 33342 in PBS and incubated at room temperature (RT) for 30 min. Liquid was then aspirated, and

plates were then washed twice with 85 μ L PBS, leaving the final wash in the plate. The plates were then sealed with adhesive aluminum plate seals. Fluorescent images of three fields of view in each of the DAPI (Hoechst stained nuclei), FITC (TIF2-GFP), and Texas Red (AR-RFP) channels were acquired on an ImageXpress Micro (IXM) automated HCS platform (Molecular Devices, LLC, Sunnyvale, CA) using a $10\times$ Plan Fluor 0.3 NA objective. Images were then analyzed using the translocation enhanced (TE) image analysis module of the MetaXpress software as described previously.^{22–25}

TIF2 and SRC1 M2H Assays

The 5xGAL4-TATA-luciferase reporter plasmid was a kind gift from Dr. Richard Maurer from the Oregon Health and Science University,⁵⁰ and constructs pGAL4-hAR-658-919 (AR-LBD amino acids 658–919 expressed as a fusion protein with Gal4-DBD [galactose 4 DNA binding domain DNA-binding domain]),⁵¹ pVP16-SRC1 (full-length SRC1 expressed as a fusion with VP16 activation domain),⁵² and pVP16-empty vector were kindly provided to us by Dr. Elizabeth Wilson, from UNC Chapel Hill. pVP16-TIF2 was generated by ligating TIF2, polymerase chain reaction (PCR) amplified from pCR-BluntII-TOPO-NCoA2 (TIF2) complementary DNA (cDNA; purchased from Dharmacon, Lafayette, CO), into the empty pVP16 vector.

HEK 293 cells were cotransfected with 5 ng of pGAL4-AR-LBD, 10 ng of either pVP16-TIF2 or pVP16-SRC1, and 20 ng of the 5xGal4-TATA-Luc reporter. Cells were transiently bulk cotransfected with the three plasmids using the FuGENE 6 transfection reagent. Before the transfection of cells, each of the pGAL4-hAR-658-919, 5xGAL4-TATA-luciferase reporter, and either pVP16-TIF2 or pVP16-SRC1 plasmid DNAs were individually incubated with FuGENE 6 at a 3:1 ratio for 25 min at RT. Each FuGENE 6 to plasmid DNA mixture was then added to HEK 293 cells that were suspended in DMEM (Cellgro 10013CV) with 2 mM L-glutamine (Invitrogen) that was supplemented with 10% FBS, and 5,000 cells in a volume of 40 μ L were seeded into the wells of 384-well assay plates and cultured overnight at 37°C , 5% CO_2 , and 95% humidity. Twenty-four hours after cell seeding into assay plates, 5 μ L of serially diluted compounds were transferred to assay wells and plates were incubated at 37°C , 5% CO_2 , and 95% humidity for 3 h before 5 μ L of 0.25 μ M DHT (25 nM final) was transferred into each well, and the assay plates were returned to the incubator for an additional 24 h. After 24 h, 25 μ L of BrightGlo[®] reagent was added to the plate and the relative light units (RLUs) were captured on a SpectraMax M5e microtiter plate reader (Molecular Devices, LLC, San Jose, CA).

PSA6.1 Promoter-Driven Luciferase Reporter Assay Developed in PC3-AR-FL-GFP Cells

The pPSA-6.1-Luc luciferase reporter plasmid was kindly provided by Dr. Zhou Wang in the Department of Urology of the University of Pittsburgh Hillman Cancer Institute.⁵³ FuGENE HD and PSA6.1-Luc were combined at a 3:1 ($\mu\text{L}:\mu\text{g}$) ratio, respectively, in Opti-MEM and incubated for 25 min at RT before being used to bulk transfect AR-FL-EGFP-PC3 cells in RPMI 1640 media containing 1% L-glutamine, and 10% FBS. Transfected cells were then seeded into white opaque 384-well assay plates (No. 781080; Greiner Bio-One) at a seeding density of 3,000 cells per well in a volume of 40 μL and incubated at 5% CO_2 , 37°C, and 95% humidity for 24 h. Each well received 20 ng of PSA6.1 LUC plasmid DNA. After 24 h, 5 μL of the serially diluted compounds was transferred to assay wells, and after a 3-h incubation, 5 μL of 0.25 μM DHT (25 nM final) in SFM was transferred to each well and assay plates were returned to the incubator for an additional 24 h before 25 μL of BrightGlo luciferase reagent (Promega) was added to the wells and the RLUs were captured on a SpectraMax M5e plate reader (Molecular Devices, LLC).

AR-LBD::TIF2-Box III-LXXLL-Peptide Binding Assay

The pET28a-AR-LBD (622–919) construct⁵⁴ was a gift from Dr. Fletterick and Dr. Nguyen of University of California San Francisco. Biotinylated-TIF2-box-III (738–756) peptide (Biotin-HN-CKKKENALLRYLLDKDDTKD-CONH₂) and non-biotinylated TIF2-box-III (738–756) peptide (H₂N-CKKKENALLRYLLDKDDTKD-CONH₂) were synthesized by the Peptide & Peptoid Synthesis Facility, at the University of Pittsburgh Health Sciences Core Research Facilities. An ALPHAScreen kit containing streptavidin donor beads (SA-DB) and nickel chelate acceptor beads (Ni-AB) was purchased from Perkin Elmer. The assay was carried out in 384-well white opaque plates (No. 781080; Greiner Bio-One). One hundred fifty nanomolars of biotinylated TIF2-box III peptide was incubated with 5 $\mu\text{g}/\mu\text{L}$ SA-DB for 30 min at RT in the dark. At the same time, His₆-AR-LBD (400 ng/well) was incubated with 10 μM DHT and 5 $\mu\text{g}/\mu\text{L}$ Ni²⁺-Abs for 30 min at RT in the dark. Around 18 μL of the SA-DB bound biotinylated TIF2 peptide mixture was added to the assay plate before 5 μL of the serially diluted compounds was transferred into assay wells and then 27 μL of the His₆-AR-LBD-Ni-DA bead mixture was added to the plate. Thirty-two wells containing 0.5% DMSO were used as maximum controls and 32 wells containing a 500-fold excess of unlabeled TIF2-box-III (75 μM) were used as minimum controls. The combined bead-protein-peptide-compound mixture was incubated for 1 h at

RT in the dark, and then, the RLUs were acquired at 520 nm after excitation at 680 nm on an Envision plate reader (Perkin Elmer).

H³-DHT Radioligand Binding Assay

The development of the His₆-AR-LBD H³-DHT competition binding assay has been described previously.²⁴ Briefly, the assay was carried out in 96-well Cu²⁺-coated plates (Thermo Fisher Scientific) that were incubated overnight at 4°C with 5 μg per well His₆-AR-LBD in 100 μL of PBS. Unbound His₆-AR-LBD was aspirated, the plate was washed 3 $\times$ with 100 μL of 0.05% Tween 20 in PBS, and then blocked with 100 μL of 1 mg/mL bovine serum albumin in PBS for 1 h. After three more washes with 100 μL of PBS and 0.05% Tween 20, 40 μL of PBS was added to the wells using a Matrix pipettor. Next, 5 μL of diluted compound followed by 5 μL of 100 nM H³-DHT were transferred into the wells using a Matrix pipettor. Compounds were tested in a 10-point twofold serial dilution series ranging between 0.098 and 50 μM in the presence of 10 nM H³-DHT. After a 1-h incubation, compounds and H³-DHT were aspirated and washed three times with 0.05% Tween 20 in PBS; 100 μL of MicroscintTM-20 micro-scintillation cocktail buffer (Perkin Elmer) was added to each well, plates were sealed with adhesive plastic covers; and the counts per minute (CPMs) were captured in a TopCount NXT microtiter plate reader (Perkin Elmer).

pGal4-AR-NTD Transactivation Assay

The 5xGAL4-TATA-luciferase reporter plasmid was provided by Dr. Richard Maurer,⁵⁰ as described above. AR NTD constructs pGAL4-hAR-1-503, AR amino acids 1–503 expressed as a fusion protein with Gal4-DBD,⁵¹ pVP16-SRC1 (full-length SRC1 expressed as a fusion with VP16 activation domain),⁵² and pVP16-empty vector were kindly provided to us by Dr. Elizabeth Wilson, from the University of North Carolina Chapel Hill. The pVP16-TIF2 construct was generated as described above. An empty pGALO vector was kindly provided by Dr. Gordon Tomaselli (Albert Einstein College of Medicine), and pGAL-hAR-1-558 and pGAL4-AR3 (AR-V7) were generated by ligating PCR-amplified inserts into the pGALO M2H vector. PCR-amplified inserts were cut with *Bam*HI and *Xba*I restriction enzymes. Primers were synthesized and purchased from Thermo Fisher Scientific.

HEK 293 cells were transiently bulk cotransfected with 5 ng of the pGal4-AR-NTD (1–503) and 20 ng of the 5xGal4-TATA-Luc reporter plasmids using the FuGENE 6 transfection reagent (Promega). Before the cotransfection of cells, the pGAL4-hAR-1-503 and 5xGAL4-TATA-luciferase reporter

plasmids were individually incubated with FuGENE 6 at a 3:1 ratio for 25 min at RT and the mixture was then added to HEK 293 cells that were suspended in DMEM (Cellgro10013CV) with 2 mM L-glutamine (Invitrogen), which was supplemented with 10% FBS (Gemini Bio-Products). Five thousand cells in a volume of 40 μ L were then seeded into the wells of 384-well assay plates and cultured overnight at 37°C, 5% CO₂, and 95% humidity. Twenty-four hours after cell seeding, 10 μ L of serially diluted compounds was transferred to the wells and assay plates were returned to the incubator for an additional 24 h before 25 μ L of BrightGlo reagent was added to the plate wells, and the RLUs were captured on a SpectraMax M5e microtiter plate reader.

PSA6.1 Promoter-Driven Luciferase Reporter Assay in PC3-AR-V7-GFP Cells

PC3-AR-V7-GFP cells were transfected with a mixture of FuGENE HD and the PSA-6.1-Luc reporter plasmid combined at a 3:1 (μ L: μ g) ratio in Opti-MEM (Gibco), which had been incubated for 25 min at RT before being added to cells that were suspended in RPMI 1640 (Gibco) media containing 1% L-glutamine (Invitrogen) and 10% FBS (Gemini Bio-Products). Bulk transfected PC3-AR-V7-GFP cells were then seeded into white opaque 384-well assay plates (No. 781080; Greiner Bio-One) at a seeding density of 3,000 cells per well in a volume of 40 μ L and incubated at 5% CO₂, 37°C, and 95% humidity for 24 h. Each well received 20 ng of PSA-6.1 LUC plasmid DNA. After 24 h, 5 μ L of the serially diluted compounds were transferred to assay wells and the plates were returned to the incubator for an additional 24 h before 25 μ L of BrightGlo luciferase reagent (Promega) was added to the wells, and the RLUs were captured on a SpectraMax M5e plate reader (Molecular Devices, LLC).

UBE2C Promoter-Driven Luciferase Reporter Assay in PC3-AR-V7-GFP Cells

The pGL4.28-UBE2C 20bpX3 luciferase reporter plasmid⁵⁵ was kindly provided by Dr. Yan Dong from Tulane University. FuGENE HD and the UBE2C-Luc plasmid were combined at a 3:1 (μ L: μ g) ratio in Opti-MEM and incubated for 25 min at RT before being added to PC3-AR-V7-GFP cells that were suspended in RPMI 1640 (Gibco) media containing 1% L-glutamine (Invitrogen) and 10% FBS (Gemini Bio-Products). Bulk transfected PC3-AR-V7-GFP cells were then seeded into white opaque 384-well assay plates (No. 781080; Greiner Bio-One) at a seeding density of 3,000 cell per well in a volume of 40 μ L and incubated at 5% CO₂, 37°C, and 95% humidity for 24 h. Each well received 10 ng of UBE2C-LUC plasmid DNA. After 24 h, 10 μ L of the serially diluted compounds were

transferred to assay wells and the plates were returned to the incubator for an additional 24 h before 25 μ L of BrightGlo luciferase reagent (Promega) was added to the wells, and the RLUs were captured on a SpectraMax M5e plate reader (Molecular Devices, LLC).

RESULTS

How Did the Test Compounds Perform in the AR-TIF2 PPIB HCS Assay?

We first wanted to benchmark how the selected test compounds would perform in the AR-TIF2 HCS biosensor assay (Fig. 1, *Supplementary Figs. S1 and S2*, and *Table 1*). In two independent assay runs, the DHT-induced AR-TIF2 PPI formation (C then D) format produced Z-factor coefficients ≥ 0.53 and signal-to-background (S:B) ratios ≥ 9.5 , and the format designed to identify disruptors of pre-existing AR-TIF2 PPIs (D then C) produced Z-factor coefficients ≥ 0.51 and S:B ratios ≥ 21.7 (*Supplementary Table S1*). As expected, 3-h pre-exposure to AR antagonists inhibited DHT-induced AR-TIF2 PPI formation in a concentration-dependent manner, with flutamide, bicalutamide, and enzalutamide exhibiting IC₅₀s of 8.77 ± 1.59 , 2.49 ± 0.20 , and 1.44 ± 0.16 μ M, respectively (Fig. 1A and *Table 1*). Interestingly, flutamide, bicalutamide, and enzalutamide also disrupted pre-existing AR-TIF2 PPI complexes with IC₅₀s of 20.6 ± 4.46 , 4.01 ± 1.37 , and 2.71 ± 0.27 μ M, respectively (Fig. 1D and *Table 1*). Consistent with our previous observations, the Hsp90 inhibitor 17-AAG produced an IC₅₀ of 0.20 ± 0.01 μ M in the AR-TIF2 PPI formation format, and an IC₅₀ of 0.39 ± 0.01 μ M in the disruption of pre-existing AR-TIF2 PPIs format (Fig. 1C, F, and *Table 1*). In contrast, neither of the NH₂-terminus acting compounds nor the Cyp17a1 inhibitor abiraterone acetate produced calculable IC₅₀s in either format of the AR-TIF2 PPIB assay at ≤ 50 μ M (Fig. 1B, C, E, F, and *Table 1*). None of the test compounds significantly reduced the number of U-2 OS cells (Hoechst-stained nuclei) detected in DAPI channel images, indicating that during a 3-h exposure period, they were not acutely cytotoxic at the concentrations tested.

Do Test Compounds Inhibit the TIF2 or SRC1 AR-LBD M2H Assays?

We developed M2H assays to measure the interactions between the AF-2 surface formed by DHT binding to the AR-LBD with two coactivators, either TIF2 or SRC1. The M2H assays were developed in the HEK 293 cell background transiently transfected with either full-length TIF2 or SRC1 chimeras attached to the VP16 transcriptional activation domain, AR-LBD fused to the Gal4 DNA binding domain, and the 5xGal4-TATA-Luc reporter construct (*Supplementary Fig. S3A*). To

Fig. 1. Inhibition of DHT-induced AR-TIF2 PPI formation (A–C) or disruption of preformed AR-TIF2 PPI complexes (D–F). U-2 OS cells were coinfecting with AR-RFP and TIF2-GFP rAV biosensors, and 3,000 cells were seeded into the wells of 384-well assay plates and cultured overnight at 37°C, 5% CO₂, and 95% humidity. After 24 h, test compounds and DHT were added to cells in two formats. To determine if test compounds could inhibit DHT-induced AR-TIF2 PPI formation (A–C), coinfecting U-2 OS cells were exposed to compounds at the indicated concentrations for 3 h before treatment with 25 nM DHT for 90 min; (A) (red-filled circle) flutamide, (blue-filled square) bicalutamide, and (green-filled triangle) enzalutamide, (B) (red-filled circle) compound No. 10, (blue-filled square) EPI-001, (C) (red-filled circle) 17-AAG, and (blue-filled square) abiraterone acetate. To determine if test compounds could disrupt preformed AR-TIF2 PPI complexes (D–F), coinfecting U-2 OS cells were exposed to 25 nM of DHT for 90 min before treatment with the indicated concentrations of compounds for 3 h; (D) (red-filled circle) flutamide, (blue-filled square) bicalutamide, and (green-filled triangle) enzalutamide, (E) (red-filled circle) compound No. 10, (blue-filled square) EPI-001, (F) (red-filled circle) 17-AAG, and (blue-filled square) abiraterone acetate. The AR-TIF2 PPI data were normalized to maximum (25 nM DHT + 0.5% DMSO, $n = 32$) and minimum (0.5% DMSO, $n = 32$) plate controls, and the percent inhibition was plotted as the mean $\pm$ SD ($n = 3$) values from triplicate wells for each compound concentration. Representative experimental data from one of two independent experiments are shown. 17-AAG, 17-allylaminogeldanamycin; AR, androgen receptor; DHT, dihydrotestosterone; DMSO, dimethyl sulfoxide; GFP, green fluorescent protein; PPI, protein–protein interaction; rAV, recombinant adenovirus; RFP, red fluorescent protein; SD, standard deviation; TIF2, transcriptional intermediary factor 2; U-2 OS, U-2 osteosarcoma cell line.

determine the optimal amounts of pGal4-AR-LBD, pVP16-TIF2, or pVP16-SRC1 plasmids, we performed cross-titration transient transfection experiments with increasing amounts of the pGal4-AR-LBD and pVP16-coactivator plasmids at a fixed amount (20 ng) of 5xGal4-TATA-Luc reporter construct (Supplementary Fig. S3B, E). Only HEK 293 cells that were

cotransfected with all three plasmids and were exposed to DHT exhibited a luciferase response substantially greater than background. In both the TIF2 and SRC1 M2H assays, the DHT-induced luciferase response increased in a linear manner as the amount of pGal4-AR-LBD cotransfected into the HEK 293 cells increased. Similarly, the DHT-induced M2H luciferase

Table 1. Test Compound 50% Inhibition Concentration Summary in AR-TIF2 Protein-Protein Interaction Biosensor

Test compound	AR-TIF2 PPIB IC ₅₀ (μM)					
	C then D ^a		D then C ^b		U-2 OS Cytotoxic	
	Mean	SD	Mean	SD	Mean	SD
Flutamide	8.77	1.59	20.6	4.46	>50	N/A
Bicalutamide	2.49	0.2	4.01	1.37	>50	N/A
Enzalutamide	1.44	0.16	2.71	0.27	>50	N/A
Compound-10	>50	N/A	>50	N/A	>50	N/A
EPI-001	>50	N/A	>50	N/A	>50	N/A
17-AAG	0.2	0.02	0.39	0.01	>50	N/A
Abiraterone	>50	N/A	>50	N/A	>50	N/A

^aPretreatment with compound before DHT addition.^bPretreatment with DHT before compound addition.17-AAG, 17-allylaminogeldanamycin; AR, androgen receptor; Cytotoxic, cytotoxicity; DHT, dihydrotestosterone; IC₅₀, 50% inhibition concentration; N/A, not applicable; PPIB, protein-protein interaction biosensor; SD, standard deviation; TIF2, transcriptional intermediary factor 2; U-2 OS, U-2 osteosarcoma cell line.

response increased in a roughly linear manner as the amount of the pVP16-coactivator constructs cotransfected into the HEK 293 cells increased. The magnitude of the TIF2 M2H response was ~30-fold higher than the signal in the SRC1 M2H assay (*Supplementary Fig. S3B* versus *E*). The final amounts of plasmid DNAs selected for the optimized M2H assays were 5 ng for pGal-AR-LBD, 10 ng each of the pVP16-coactivator constructs, and 20 ng of the 5xGal4-TATA-Luc reporter construct. A DHT concentration response was performed to determine the optimal concentration of DHT to use in subsequent M2H experiments. DHT exhibited EC₅₀s of 0.619 and 2.23 nM for the TIF2 and SRC1 M2H assays, respectively (*Supplementary Fig. S3C, F*), and we selected 25 nM DHT as a suitable agonist concentration for activating both M2H assay formats. DMSO tolerance experiments in the TIF2 and SRC1 M2H formats indicated that 0.5% DMSO would not interfere in either assay (*Supplementary Fig. S3D, G*).

Both M2H assays performed well in two independent compound testing runs with the SRC1 AR-LBD assay producing Z-factor coefficients ≥0.47 and S:B ratios ≥10.4, and the TIF2 AR-LBD assay producing Z-factor coefficients ≥0.48 and S:B ratios ≥45.6 (*Supplementary Table S1*). All seven test compounds inhibited DHT-induced AR-LBD interactions with the TIF2 and SRC1 coactivators in a concentration-dependent manner (*Figs. 2* and *3*). In the TIF2 M2H assay, the anti-

androgens flutamide, bicalutamide, and enzalutamide exhibited IC₅₀s of 3.01 ± 0.75, 2.01 ± 0.99, and 2.36 ± 1.44 μM, respectively, and the Hsp90 inhibitor 17-AAG produced an IC₅₀ of 0.33 ± 0.02 μM (*Fig. 2A* and *Table 2*). In the SRC1 M2H assay, flutamide, bicalutamide, and enzalutamide exhibited IC₅₀s of 2.53 ± 0.66, 1.49 ± 0.69, and 1.37 ± 0.91 μM, respectively (*Fig. 3A* and *Table 2*), and 17-AAG produced an IC₅₀ of 0.45 ± 0.08 μM (*Fig. 3C* and *Table 2*). None of these four test compounds produced substantial levels of cytotoxicity in either M2H assay (*Figs. 2D, 2F, 3D, 3F*, and *Table 2*). Although compound No. 10, EPI-001, and abiraterone acetate also inhibited both M2H assays, they likewise displayed substantial concentration-dependent cytotoxicity in HEK 293 cells (*Figs. 2B, 2C, 2E, 2F, 3B, 3C, 3E, 3F*, and *Table 2*). Indeed, compound-induced cytotoxicity may be the primary mechanism of decreasing the M2H reporter signals by compound No. 10, EPI-001, and abiraterone acetate (*Figs. 2E, 2F, 3E, 3F*, and *Table 2*). Overall, none of the test compounds were selective at disrupting DHT-induced AR-LBD interactions with either TIF2 or SRC1.

Do Test Compounds Inhibit the PSA6.1 Promoter-Driven Luciferase Reporter Assay Conducted in PC3 Cells Stably Expressing AR-FL-EGFP?

We wanted to examine whether test compounds could block the transcriptional activity of AR-FL expressed in a PCa cell background. The PSA6.1 luciferase reporter is controlled by a fragment of the PSA promoter that contains three AREs fused to a luciferase reporter gene.⁵³ First, we generated a cell line that stably expresses AR-FL-EGFP in the AR null PC3 PCa cell line. *Supplementary Figure S4A* shows representative grayscale DAPI and FITC channel and color composite images of the AR-FL-EGFP-PC3 cell line ± DHT exposure, which were stained with the Hoechst DNA stain. For comparison, images of the AR null parental PC3 cell line are also presented. Exposure to DHT alters the subcellular localization of the AR-FL-EGFP to a predominantly nuclear distribution in images captured on the IXM HCS platform (*Supplementary Fig. S4A*). Exposure of AR-FL-EGFP-PC3 cells to DHT increased the inner/outer intensity ratio output of the TE image analysis module, which is a measure of the mean average fluorescence intensity of AR-FL-GFP in the nucleus, divided by the mean average fluorescence intensity of AR-FL-GFP in the cytoplasm. In PC3-AR-FL-EGFP cells, DHT induced a concentration-dependent increase in the inner/outer intensity ratio with an EC₅₀ of 1.23 ± 0.131 nM (*Supplementary Fig. S4B*). After PC3-AR-FL-EGFP cells were transiently transfected with the PSA6.1-Luc reporter construct, DHT induced a concentration-dependent increase in

Fig. 2. Inhibition of DHT-induced M2H Interactions between AR-LBD and TIF2. HEK 293 cells that were cotransfected with three plasmid constructs, pGAL4-AR-LBD, 5xGal4-TATA-Luc, and pVP16-TIF2, were seeded at 5,000 cells per well in 384-well assay plates, and cultured overnight at 37°C, 5% CO₂, and 95% humidity. After 24 h, cells were exposed to test compounds at the indicated concentrations for 3 h before the addition of 25 nM DHT. After an additional 24-h incubation, BrightGlo[®] reagent was added to the assay plates and the RLUs were captured on a SpectraMax M5e microtiter plate reader. The M2H RLU's data were normalized to maximum (25 nM DHT + 0.5% DMSO, $n=32$) and minimum (0.5% DMSO, $n=32$) plate controls, and the percent inhibition was plotted as the mean $\pm$ SD ($n=3$) values from triplicate wells for each compound concentration. The normalized percent inhibition of the DHT-induced AR-LBD TIF2 M2H responses for (A) (red-filled circle) flutamide, (blue-filled square) bicalutamide, and (green-filled triangle) enzalutamide, (B) (red-filled circle) compound No. 10, (blue-filled square) EPI-001, (C) (red-filled circle) 17-AAG, and (blue-filled square) abiraterone acetate are presented. The corresponding cell viability data for compound-treated wells were normalized to 0.5% DMSO control wells ($n=32$), and the percent cell viability plotted as the mean $\pm$ SD ($n=3$) values from triplicate wells for each compound concentration is presented in (D) (red open circle) flutamide, (blue open square) bicalutamide, and (green open triangle) enzalutamide, (E) (red open circle) compound No. 10, (blue open square) EPI-001, (F) (red open circle) 17-AAG, and (blue open square) abiraterone acetate. Representative experimental data from one of two independent experiments are shown. LBD, ligand-binding domain; Luc, luciferase; M2H, mammalian 2-hybrid; pGal4, DNA-binding domain of the Gal4 protein derived from yeast; RLUs, relative light units.

reporter activity with an EC₅₀ of 0.280 ± 0.034 nM (Supplementary Fig. S4C). We selected 25 nM DHT as a suitable agonist concentration for conducting the PSA6.1-Luc reporter assay in the PC3-AR-FL-EGFP cell background.

In independent compound testing runs in the PC3-AR-FL-EGFP-PSA-6.1-Luc reporter assay, the assay performed well with Z-factor coefficients ≥ 0.40 and S:B ratios ≥ 5.6 (Supplementary Table S1). As anticipated, the antiandrogens flutamide, bicalutamide, and enzalutamide inhibited DHT-induced reporter activity in a concentration-dependent

manner, exhibiting IC₅₀s of 17.4 ± 3.91 , 21.9 ± 4.80 , and 19.5 ± 3.45 μ M, respectively (Fig. 4B and Table 2). Similarly, the Hsp90 inhibitor 17-AAG produced an IC₅₀ of 1.72 ± 0.48 μ M (Fig. 4D and Table 2). In contrast, compound No. 10, EPI-001 and abiraterone acetate failed to inhibit DHT-induced transcriptional activity in PC3-AR-FL-EGFP cells at ≤ 50 μ M (Fig. 4C, D, and Table 2). None of the test compounds exhibited evidence of cytotoxicity in PC3-AR-FL-EGFP cell line viability assays conducted in parallel with the reporter assays (Table 2).

Fig. 3. Inhibition of DHT-induced M2H Interactions between AR-LBD and SRC1. HEK 293 cells that were cotransfected with three plasmid constructs, pGAL4-AR-LBD plus 5xGal4-TATA-Luc with pVP16-SRC1, were seeded at 5,000 cells per well in 384-well assay plates, and cultured overnight at 37°C, 5% CO₂, and 95% humidity. After 24 h, cells were exposed to test compounds at the indicated concentrations for 3 h before the addition of 25 nM DHT. After an additional 24-h incubation, BrightGlo reagent was added to the assay plates and the RLUs were captured on a SpectraMax M5e microtiter plate reader. The M2H RLU's data were normalized to maximum (25 nM DHT + 0.5% DMSO, $n=32$) and minimum (0.5% DMSO, $n=32$) plate controls, and the percent inhibition was plotted as the mean $\pm$ SD ($n=3$) values from triplicate wells for each compound concentration. The normalized percent inhibition of the DHT-induced AR-LBD SRC1 M2H responses for (A) (red-filled circle) flutamide, (blue-filled square) bicalutamide, and (green-filled triangle) enzalutamide, (B) (red-filled circle) compound No. 10, (blue-filled square) EPI-001, (C) (red-filled circle) 17-AAG, and (blue-filled square) abiraterone acetate is presented. The corresponding cell viability data for compound-treated wells were normalized to 0.5% DMSO control wells ($n=32$), and the percent cell viability plotted as the mean $\pm$ SD ($n=3$) values from triplicate wells for each compound concentration is presented in (D) (red open circle) flutamide, (blue open square) bicalutamide, and (green open triangle) enzalutamide, (E) (red open circle) compound No. 10, (blue open square) EPI-001, (F) (red open circle) 17-AAG, and (blue open square) abiraterone acetate. Representative experimental data from one of two independent experiments are shown. SRC, steroid receptor coactivator.

Do Test Compounds Antagonize H³-DHT Binding to the AR-LBD?

The H³-DHT radioligand binding assay was incorporated into our screening paradigm to identify and deprioritize hits from the AR-TIF2 biosensor HCS assay that inhibited/disrupted AR-TIF2 PPIs through ligand binding antagonism. This assay specifically measures the displacement of H³-DHT from AR-LBD in the presence of increasing concentrations of test compounds and was performed as described previously.²⁴ The H³-DHT radioligand binding assay performed well in two

independent compound testing runs and produced Z-factor coefficients ≥ 0.79 and S:B ratios ≥ 38.0 (Supplementary Table S1). As expected, the AR antagonists flutamide, bicalutamide, and enzalutamide displaced H³-DHT binding to His₆-AR-LBD with IC₅₀ values of 48.6 ± 19.5 , 5.53 ± 3.27 , and 50.2 ± 14.4 μ M, respectively (Fig. 5A and Table 2). Since none of the other test compounds interact with the AR ligand binding site, it was reassuring that they failed to displace H³-DHT binding to His₆-AR-LBD at ≤ 50 μ M (Fig. 5B, C, and Table 2).

Table 2. Test Compound 50% Inhibition Concentration Summary in Activation Function 2 Transactivation Domain-Focused Assays

Assays	Mammalian 2-hybrid IC ₅₀ (μM)						PSA6.1-Luc reporter IC ₅₀ (μM)				LXXLL-AR-LBD binding IC ₅₀ (μM)		H ³ -DHT binding IC ₅₀ (μM)	
	TIF2		SRC1		HEK 293 cell Cytos		PC3-AR-GFP		Cytos		TIF2 Box III LXXLL		AR-LBD	
Test compound	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Flutamide	3.01	0.75	2.53	0.66	>50	N/A	17.4	3.91	>50	N/A	>50	N/A	48.6	19.5
Bicalutamide	2.01	0.99	1.49	0.69	>50	N/A	21.9	4.8	>50	N/A	>50	N/A	5.53	3.27
Enzalutamide	2.36	1.44	1.37	0.91	>50	N/A	19.5	3.45	>50	N/A	>50	N/A	50.2	14.4
Compound-10	2.32	0.91	1.99	0.19	26.5	10.9	>50	N/A	>50	N/A	>50	N/A	>50	N/A
EPI-001	9.15	2.47	8.15	1.27	36.6	9.3	>50	N/A	>50	N/A	>50	N/A	>50	N/A
17-AAG	0.33	0.02	0.45	0.08	>50	N/A	1.72	0.48	>50	N/A	29.8	9.78	>50	N/A
Abiraterone	4.47	1.96	2.43	0.64	30.5	7.72	>50	N/A	>50	N/A	>50	N/A	>50	N/A

AR-LBD, ligand-binding domain of the androgen receptor; GFP, green fluorescent protein; H³-DHT, tritiated dihydrotestosterone; HEK 293 cells, human embryonic kidney 293 cells; Luc, luciferase; LXXLL, coactivator leucine rich binding motifs; PC3, human prostate cancer cell line; PSA, prostate-specific antigen; SRC1, steroid receptor coactivator 1.

Do Test Compounds Inhibit TIF2 Box III LXXLL Peptide Binding to the AR-LBD?

We developed a biochemical assay to measure the binding interactions between the AR AF-2 surface formed by the DHT-activated AR-LBD and a peptide containing the LXXLL motif located in box III of the TIF2 coactivator's nuclear interaction domain (NID). It has been reported that the LXXLL motif from box III of the TIF2 NID contributes most of the binding interactions with the AR AF-2 surface.^{37,39,56,57} In our assay, the histidine-tagged AR-LBD protein was chelated to Ni²⁺-coated acceptor ALPHAScreen beads, and the biotinylated TIF2 Box III LXXLL peptide was bound to SA-DB. Binding of the two interacting partners promotes proximity between the donor and acceptor beads, which can be quantified through increased light emission at a wavelength of 520–620 nm after excitation at 680 nm⁵⁸ (*Supplementary Fig. S5A*). To determine the optimal amounts of the His₆-AR-LBD protein and biotin-TIF2-LXXLL-peptide to use in the ALPHAScreen assay, we performed titration experiments (*Supplementary Fig. S5B, C*). Based on the data from these experiments, we selected 400 ng/well of the His₆-AR-LBD protein and 150 nM biotin-TIF2-LXXLL-peptide for the optimized ALPHAScreen assay conditions. Under these conditions, the apparent K_d for the biotin-TIF2-LXXLL-peptide binding to His₆-AR-LBD was 151.3 nM, and the B_{max} ALPHAScreen signal was extrapolated at 42,880 CPMs (*Supplementary Fig. S5C*).

In competitive displacement binding experiments, non-biotinylated TIF2-LXXLL-peptide exhibited an IC₅₀ of 2.76 nM for the displacement of biotin-TIF2-LXXLL-peptide binding from His₆-AR-LBD (*Fig. 6A*). The biotin-TIF2-LXXLL-peptide::AR-LBD ALPHAScreen assay performed well in two independent test compound runs and produced Z-factor coefficients ≥0.74 and S:B ratios ≥33.4 (*Supplementary Table S1*). We did not anticipate that any of the test compounds would inhibit or disrupt LXXLL motif binding to the AF-2 surface because they either bind to the ligand binding site or NTD of AR, or to other protein targets (Hsp90 or Cyp17a1). Surprisingly, the Hsp90 inhibitor 17-AAG exhibited an IC₅₀ of 29.8 ± 9.78 μM in the biotin-TIF2-LXXLL-peptide::AR-LBD binding assay, and although the antiandrogen flutamide failed to achieve 50% inhibition at ≤50 μM, it also appeared to inhibit peptide binding in a concentration-dependent manner (*Fig. 6B, D, and Table 2*). None of the other test compounds inhibited biotin-TIF2-LXXLL-peptide binding to AR-LBD (*Fig. 6B–D and Table 2*).

Can Any of the Test Compounds Block AR-NTD (AF-1's) Transcriptional Activity?

We initially set out to develop AF-1 M2H assays by substituting a Gal4-AR-NTD construct for the Gal4-AR-LBD construct used in the M2H assays described above (*Supplementary Fig. S6A*). However, not only was Gal4-AR-NTD-mediated transcriptional activity independent of DHT treatment

Fig. 4: Inhibition of DHT-induced PSA6.1-luciferase reporter assay in PC3-AR-FL-GFP cells. PC3-AR-FL-GFP cell lines were transiently bulk transfected with a 3:1 ratio of FUGENETM HD: PSA-6.1-Luc plasmid DNA (20 ng/well) and then seeded into 384-well assay plates at a density of 3,000 cell per well, and incubated overnight at 5% CO₂, 95% humidity, and 37°C. Transfected cells were pre-exposed to the indicated concentrations of compounds for 3 h before the addition of 25 nM DHT and the assay plate was returned to the incubator for an additional 24 h before BrightGlo reagent was added to the plate and the RLUs were captured on a SpectraMax M5e microtiter plate reader. The RLU data were normalized to maximum (25 nM DHT +0.5% DMSO, $n=32$) and minimum (0.5% DMSO, $n=32$) plate controls, and the percent inhibition was plotted as the mean $\pm$ SD ($n=3$) values from triplicate wells for each compound concentration. **(A)** The mean plate control RLU's $\pm$ SD ($n=32$) for parental PC3 cells and PC3-AR-FL-GFP cells transiently transfected with the PSA6.1-Luc reporter exposed to DMSO (black bars) or DMSO+DHT (gray bars) is presented. The normalized percent inhibition of the DHT-induced PSA6.1-Luc reporter activity for **(B)** (red-filled circle) flutamide, (blue-filled square) bicalutamide, and (green-filled triangle) enzalutamide, **(C)** (red-filled circle) compound No. 10, (blue-filled square) EPI-001, **(D)** (red-filled circle) 17-AAG, and (blue-filled square) abiraterone acetate is presented. Representative experimental data from one of two independent experiments are shown. FL, full length; PSA, prostate-specific antigen.

Fig. 5. Inhibition of AR-LBD H³-DHT Binding. Competitive displacement binding of H³-DHT to His₆-AR-LBD. Five micrograms of His₆-AR-LBD protein was added to the wells of 96-well copper-coated plates and allowed to bind overnight at 4°C. Unbound protein was removed by aspiration; the plate was washed three times with PBS-Tween 20 buffer and blocked for 1 h with 1 mg/mL BSA in PBS-Tween 20. After three washes with PBS-Tween 20 buffer, 10 nM H³-DHT was added to the wells of the plate in the presence or absence of the indicated concentrations of test compounds and incubated at RT for 1 h. Unbound H³-DHT was removed by aspiration and washing, and microscintillation reagent was added to the wells, and the CPMs were captured on a TopCount NXT microtiter β-counter. CPM data were normalized to maximum (0.5% DMSO, *n* = 8) and minimum (10 μM cold DHT, *n* = 8) plate controls, and the percent inhibition was plotted as the mean ± SD (*n* = 2) values from duplicate wells for each compound concentration. The normalized percent inhibition of the H³-DHT bound to His₆-AR-LBD for (A) (blue-filled circle) flutamide, (red-filled square) bicalutamide, and (green-filled triangle) enzalutamide, (B) (blue-filled circle) compound No. 10, (red-filled square) EPI-001, (C) (blue-filled circle) 17-AAG, and (red-filled square) abiraterone acetate is presented. Representative experimental data from one of two independent experiments are shown. BSA, bovine serum albumin; CPMs, counts per minute; PBS, phosphate-buffered saline; RT, room temperature.

as expected but it also displayed no apparent requirement for coactivation by either VP16-TIF2 or VP16-SRC1 (*Supplementary Fig. S6B*). The assay was instead used to interrogate whether compounds could block the constitutive transactivation of AR-NTD₁₋₅₀₃. In two independent test compound runs, the pGal4-AR-NTD transactivation assay maximum (DMSO) controls exhibited percent coefficient of variance (% CVs) ≤ 8.45 (*Supplementary Table S1*). All seven test compounds inhibited the constitutive transactivation of the 5xGal4-TATA-Luc reporter by GAL4-AR-NTD₁₋₅₀₃ (*Fig. 7*). Surprisingly, flutamide, bicalutamide, and enzalutamide exhibited IC₅₀s of 4.83 ± 0.14, 18.83 ± 2.46, and 31.50 ± 2.63 μM, respectively, in the NTD transactivation assay, with little or no apparent cytotoxicity (*Fig. 7A, D, and Table 3*). Similarly, 17-AAG produced an IC₅₀ of 3.54 ± 0.54 μM, also with little apparent cytotoxicity (*Fig. 7C, F, and Table 3*). Although abiraterone acetate produced an IC₅₀ of 6.43 ± 0.28 μM in the NTD transactivation assay, it also was cytotoxic with an IC₅₀ of 30.5 ± 7.72 μM (*Fig. 7C, F, and Table 3*). Compound No. 10 and EPI-001 also produced IC₅₀s of 1.55 ± 0.04 μM and 9.92 ± 1.24 μM, respectively, in the NTD transactivation assay (*Fig. 7B and Table 3*), with corresponding IC₅₀s of 26.5 ± 10.9 μM and 36.6 ± 9.3 μM, respectively, in the associated viability assays, suggesting that compound-mediated cyto-

toxicity may be contributing significantly to the decreased reporter signals (*Fig. 7E and Table 3*).

Do Any of the Test Compounds Inhibit the Constitutive Activity of the PSA-6.1 Promoter Luciferase Reporter Directed by AR-V7?

The PSA6.1 promoter-driven luciferase reporter can be activated by both AR-FL and AR-V7 splice variants that are expressed in PCa cells.²⁴ To provide an AF-1-mediated transcriptional activation assay, we transfected the PSA6.1-Luc reporter construct into the AR null PC3 cell line that had been transformed to express AR-V7-EGFP. *Supplementary Figure S7A* shows representative grayscale DAPI and FITC channel and color composite images of the AR-V7-EGFP-PC3 cell line±DHT exposure stained with the Hoechst DNA stain. For comparison, images of the AR null parental PC3 cell line are also presented. Exposure to DHT had no effect on the subcellular localization of the AR-V7-EGFP, which is predominantly nuclear. To compare the PSA6.1 promoter-driven luciferase reporter responses in three distinct cell backgrounds, parental PC3, PC3-AR-FL-EGFP, and PC3-AR-V7-EGFP cells were transiently transfected with the PSA6.1-Luc reporter construct, cultured overnight, and then exposed to either DHT (100 nM) or DMSO for 24 h. In parental PC3 cells

Fig. 6. Inhibition of AR-LBD TIF2 Box III LXXLL peptide binding. Biotinylated TIF2 peptide was incubated with streptavidin donor beads for 30 min in the dark at RT, and then added to 384-well assay plates at a final concentration of 150 nM. Compounds at the indicated concentrations were then added to the wells of the plate, which was incubated for 30 min in the dark at RT. Simultaneously, pET28a-His₆-AR-LBD (400 ng/well) was incubated with DHT and Ni²⁺-coated acceptor beads for 30 min and then this mixture was added to the wells of the 384-well plates. After mixing the ALPHA Screen components, the assay plates were incubated for 1 h at RT in the dark and the CPMs were captured on an Envision plate reader at 520 nm after excitation at 680 nm. **(A)** Competitive displacement binding of biotinylated-TIF2-box-III LXXLL peptide to His₆-AR-LBD by unlabeled TIF2-box-III LXXLL peptide. The CPM data were normalized to maximum (0.5% DMSO, $n=32$) and minimum (0.5% DMSO and 75 μM unlabeled TIF2, $n=32$) plate controls, and the percent inhibition was plotted as the mean $\pm$ SD ($n=3$) values from triplicate wells for each compound concentration. The normalized percent inhibition of the AR-LBD::LXXLL-peptide binding for **(B)** (red-filled circle) flutamide, (blue-filled square) abiraterone bicalutamide, and (green-filled triangle) enzalutamide, **(C)** (red-filled circle) compound No. 10, (blue-filled square) EPI-001, and (blue-filled square) abiraterone acetate is presented. Representative experimental data from one of two independent experiments are shown. LXXLL, coactivator leucine-rich binding motifs.

Fig. 7. Inhibition of Constitutive pGal4-AR-NTD Transactivation. HEK 293 cells were cotransfected with two plasmid constructs, pGal4-AR-NTD (1–503) and the 5xGal4-TATA-Luc reporter, and 5,000 cells were seeded into the wells of 384-well assay plates, and cultured overnight at 37°C, 5% CO₂, and 95% humidity. After 24 h, cells were exposed to test compounds at the indicated concentrations and assay plates were returned to the incubator. After an additional 24 h, BrightGlo reagent was added to the plate and the RLUs were captured on a SpectraMax M5e microtiter plate reader. The RLU's data were normalized relative to the maximum (0.5% DMSO, $n=64$) plate controls, and the percent inhibition was plotted as the mean $\pm$ SD ($n=3$) values from triplicate wells for each compound concentration. The normalized percent inhibition for treatment with (A) (red-filled circle) flutamide, (blue-filled square) bicalutamide, and (green-filled triangle) enzalutamide, (B) (red-filled circle) compound No. 10, (blue-filled square) EPI-001, (C) (red-filled circle) 17-AAG, and (blue-filled square) abiraterone acetate is presented. The corresponding cell viability data for compound-treated wells were normalized to 0.5% DMSO control wells ($n=64$), and the percent cell viability plotted as the mean $\pm$ SD ($n=3$) values from triplicate wells at each compound concentration for (D) (red open circle) flutamide, (blue open square) bicalutamide, and (green open triangle) enzalutamide, (E) (red open circle) compound No. 10, (blue open square) EPI-001, (F) (red open circle) 17-AAG, and (blue open square) abiraterone acetate is presented. Representative experimental data from one of two independent experiments are shown. AR-NTD, N-terminal domain of AR.

that are null for AR, there was no PSA6.1-Luc reporter response with or without DHT (Supplementary Fig. S7B). In PC3-AR-FL-GFP cells, a strong PSA6.1-Luc reporter response was only observed in cells treated with DHT, whereas in PC3-AR-V7-GFP cells, robust constitutive PSA6.1-Luc reporter activity was observed with or without DHT treatment (Supplementary Fig. S7B).

In two independent test compound runs, the PC3-AR-V7-EGFP PSA6.1-Luc reporter assay performed well and the maximum (DMSO) controls exhibited % CVs ≤ 13.1 (Supplementary Table S1). The AR antagonist flutamide in-

hibited AR-V7-driven PSA6.1-Luc reporter activity with an IC₅₀ of $11.5 \pm 1.22 \mu\text{M}$ (Fig. 8B and Table 3). In contrast, neither bicalutamide nor enzalutamide produced an IC₅₀ at $\leq 50 \mu\text{M}$ (Fig. 8B and Table 3). Abiraterone acetate also failed to achieve an IC₅₀ at $\leq 50 \mu\text{M}$ (Fig. 8D and Table 3). 17-AAG produced an IC₅₀ of $0.68 \pm 0.37 \mu\text{M}$ (Fig. 8D and Table 3), and compound No. 10 and EPI-001 exhibited IC₅₀s of 50.3 ± 11.3 and $25.3 \pm 4.05 \mu\text{M}$, respectively (Fig. 8C and Table 3). None of the test compounds were cytotoxic to PC3-AR-V7-EGFP cells under the conditions used (Table 3).

Table 3. Test Compound 50% Inhibition Concentration Summary in Activation Function 1 Transactivation Domain-Focused Assays

Test Compound	Transactivation assay IC ₅₀ (μM)				PSA6.1-Luc Reporter IC ₅₀ (μM)		UBE2C-Luc Reporter IC ₅₀ (μM)		Cytotoxicity	
	pGal4-AR-NTD (1–503)		HEK 293 cell Cytotoxic		PC3-AR-V7-GFP		PC3-AR-V7-GFP		PC3-AR-V7-GFP	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Flutamide	4.83	0.14	>50	N/A	11.5	1.22	28.6	2.71	>50	N/A
Bicalutamide	18.83	2.46	>50	N/A	>50	N/A	>50	N/A	>50	N/A
Enzalutamide	31.5	2.63	>50	N/A	>50	N/A	>50	N/A	>50	N/A
Compound-10	1.55	0.04	26.5	10.9	50.3	11.3	58.6	0.79	>50	N/A
EPI-001	9.92	1.24	36.6	9.3	25.3	4.05	49.2	0.76	>50	N/A
17-AAG	3.54	0.54	>50	N/A	0.68	0.37	0.75	0.50	>50	N/A
Abiraterone	6.43	0.28	30.5	7.72	>50	N/A	>50	N/A	>50	N/A

AR-NTD, N-terminal domain of AR; AR-V7, androgen receptor splice variant 7; pGal4, DNA-binding domain of the Gal4 protein derived from yeast; UBE2C, ubiquitin-conjugating enzyme E2C.

Do Any of the Test Compounds Inhibit the Constitutive Activity of the UBE2C Promoter Luciferase Reporter Directed by AR-V7?

UBE2C is a specific target gene of AR splice variants.^{46,55,59} The UBE2C reporter is a luciferase construct driven by three repeats of an AR-V7-specific promoter element from the UBE2C gene.⁵⁵ To provide an AF-1 selective transcriptional activation assay, we transfected the UBE2C-Luc reporter construct into PC3-AR-V7-EGFP cells. To compare the UBE2C promoter-driven luciferase reporter responses in three distinct cell backgrounds, parental PC3, PC3-AR-FL-EGFP, and PC3-AR-V7-EGFP cells were transiently transfected with the UBE2C-Luc reporter construct, cultured overnight, and then exposed to either DHT (100 nM) or DMSO for 24 h. In both parental PC3 and PC3-AR-FL-EGFP cells, there was no UBE2C-Luc reporter response either with or without DHT (*Supplementary Fig. S7C*). In PC3-AR-V7-EGFP cells, however, robust constitutive UBE2C-Luc reporter activity was observed with or without DHT treatment (*Supplementary Fig. S7C*).

In two independent test compound runs, the PC3-AR-V7-EGFP UBE2C-Luc reporter assay performed well and the maximum (DMSO) controls exhibited % CVs ≤11.8 (*Fig. 9* and *Supplementary Table S1*). The antiandrogen flutamide inhibited AR-V7-driven UBE2C-Luc reporter activity with an IC₅₀ value of 28.6 ± 2.71 μM, while bicalutamide and enzalutamide failed to achieve an IC₅₀ at ≤50 μM (*Fig. 9B* and *Table 3*). Abiraterone acetate also failed to achieve an IC₅₀ at ≤50 μM in the AR-V7-driven UBE2C-Luc reporter assay

(*Fig. 9D* and *Table 3*). In contrast, the Hsp90 inhibitor 17-AAG produced an IC₅₀ of 0.75 ± 0.50 μM (*Fig. 9D* and *Table 3*), and compound No. 10 and EPI-001 exhibited IC₅₀s of 58.6 ± 0.79 and 49.2 ± 0.76 μM, respectively, in the AR-V7-driven UBE2C-Luc reporter assay (*Fig. 9C* and *Table 3*). However, compound No. 10 and EPI-001 were only active at the highest concentration used in the assay. None of the test compounds were cytotoxic to PC3-AR-V7-EGFP cells under the conditions used (*Table 3*).

DISCUSSION

Increased expression of AR coactivators and AR splice variants occurs in PCa patients who have relapsed on ADT and contributes to poor CRPC patient outcomes by increasing AR transcriptional activity under androgen-depleted conditions.^{13,16,19–21} The AF-2 and AF-1 transactivation domains serve as binding surfaces for coactivator proteins that facilitate AR gene transcription.^{14,15,22–25,33–36} Coactivator interactions with both AR-FL and splice variants compensate for low cellular DHT levels after ADT treatment and result in persistent transcriptional activity.^{13–16} The AF-2 and AF-1 binding surfaces mediate androgen-dependent and androgen-independent transactivation, respectively. AR-V7 splice variants, which are upregulated in CRPC patients who have relapsed on ADT, only contain an AF-1 surface.^{14,15,34,40,42} The ideal inhibitor/disruptor of AR-coactivator PPIs for CRPC therapy would target both the AF-2 and AF-1 surfaces and effectively block transcriptional activation by both AR-FL and splice variants. We recently implemented an AR-TIF2 PPIB

Fig. 8. Inhibition of PSA6.1-Luc Reporter Activity in PC3-AR-V7-GFP Cells. The PC3-AR-V7-GFP cell line was transiently bulk transfected with a 3:1 ratio of FuGENE HD: PSA-6.1-Luc plasmid DNA (20 ng/well), then seeded at 3,000 cells per well in 384-well assay plates, and incubated overnight at 5% CO₂, 95% humidity, and 37°C. Transfected cells were then exposed to the indicated concentrations of compounds and the assay plates were returned to the incubator for an additional 24 h before BrightGlo reagent was added to the plate and the RLUs were captured on a SpectraMax M5e microtiter plate reader. **(A)** The RLUs from PC3 and PC3-AR-V7-GFPs transfected with the PSA6.1-Luc reporter plasmid and then exposed to DMSO (black bars) or DMSO+DHT (gray bars) are presented. The PSA6.1-Luc RLU data for compound-treated wells were normalized relative to maximum plate controls (0.5% DMSO, $n = 64$), and the percent inhibition was plotted as the mean $\pm$ SD ($n = 3$) values from triplicate wells at each compound concentration. The normalized percent inhibition of the AR-V7-induced PSA6.1-Luc reporter activity for **(B)** (red-filled circle) flutamide, (blue-filled square) bicalutamide, and (green-filled triangle) enzalutamide, **(C)** (red-filled circle) compound No. 10, (blue-filled square) EPI-001, **(D)** (red-filled circle) 17-AAG, and (blue-filled square) abiraterone acetate is presented. Representative experimental data from one of two independent experiments are shown. AR-V7, androgen receptor splice variant 7.

Fig. 9. Inhibition of UBE2C-Luc Reporter Activity in PC3-AR-V7-GFP Cells. The PC3-AR-V7-GFP cell line was transiently bulk transfected with a 3:1 ratio of FuGENE HD: UBE2C-Luc plasmid DNA (10 ng/well) and then seeded at 3,000 cell per well into 384-well assay plates and incubated overnight at 5% CO₂, 95% humidity, and 37°C. Transfected cells were then exposed to the indicated concentrations of compounds before the assay plate was returned to the incubator for an additional 24 h before BrightGlo reagent was added to the plate and the RLUs were captured on a SpectraMax M5e microtiter plate reader. **(A)** The RLUs from PC3 and PC3-AR-V7-GFPs transfected with the UBE2C-Luc reporter plasmid and then exposed to DMSO (black bars) or DMSO+DHT (gray bars) are presented. The UBE2C-Luc RLU data for compound-treated wells were normalized relative to maximum plate controls (0.5% DMSO, $n = 64$), and the percent inhibition was plotted as the mean $\pm$ SD ($n = 3$) values from triplicate wells at each compound concentration. The normalized percent inhibition of the DHT-induced UBE2C-Luc reporter activity for **(B)** (red-filled circle) flutamide, (blue-filled square) bicalutamide, and (green-filled triangle) enzalutamide, **(C)** (red-filled circle) compound No. 10, (blue-filled square) EPI-001, **(D)** (red-filled circle) 17-AAG, and (blue-filled square) abiraterone acetate is presented. Representative experimental data from one of two independent experiments are shown. UBE2C, ubiquitin-conjugating enzyme E2C.

HCS campaign of $\geq 143,000$ compounds to identify hits that inhibit or disrupt the interactions between AR and the TIF2 coactivator.^{22–25} Since the AR-TIF2 PPIB design incorporates only the AR-LBD region of AR and the region of the TIF2 coactivator's NID that contains the box III LXXLL motif, we were concerned that hits might be biased toward the AF-2 surface. We therefore developed a panel of AF-2- and AF-1-focused assays to characterize the transactivation domain profiles of our hits and to prioritize those that were effective at blocking coactivator recruitment at both surfaces (*Supplementary Table S2*).

To validate our AF-2 and AF-1 assays, we assembled a test set of four FDA-approved ADTs, two investigational molecules that target the AR-NTD, and an inhibitor of the Hsp90 molecular chaperone. Flutamide, bicalutamide, and enzalutamide are AR-antagonists that are prescribed as components of an ADT strategy (*Supplementary Fig. S2*).^{60–62} Similarly, the Cyp17a1 inhibitor abiraterone acetate, which blocks androgen synthesis, is also used as a component of ADT.⁶³ Compounds No. 10 and EPI-001 target the amino terminus of AR rather than the ligand binding site,^{30,41,48,49,64} and 17-AAG is an inhibitor of the Hsp90 molecular chaperone ATPase that is involved in the folding, maturation, and maintenance of AR in a high-affinity ligand binding conformation (*Supplementary Fig. S2*).⁶⁵ Consistent with previous studies,^{22–25} all three AR antagonists and the Hsp90 inhibitor 17-AAG effectively inhibited DHT-induced AR-TIF2 PPI formation and disrupted preformed AR-TIF2 complexes in a concentration-dependent manner (*Fig. 1* and *Table 1*). In contrast, compound No. 10, EPI-001, or abiraterone acetate did not produce a calculable IC_{50} in either format of the AR-TIF2 PPIB assay (*Fig. 1* and *Table 1*). Since compound No. 10 and EPI-001 target the AR-NTD, which is not part of the AR-RFP biosensor, and abiraterone acetate inhibits Cyp17a1, an enzyme involved with androgen biosynthesis, it was expected that these compounds would not be active in the AR-TIF2 biosensor. Although compound No. 10 failed to produce a calculable IC_{50} , it inhibited DHT-induced AR-TIF2 PPI formation, potentially due to its ability to reduce AR expression levels and/or restrict its localization to the cytoplasm.^{30,48,49} None of the test compounds were acutely cytotoxic toward U-2 OS cells under the conditions used.

We then profiled the activities of the test compounds in five AF-2-focused assays (*Figs. 2–6* and *Table 2*). M2H assays represent the gold standard method for measuring how NR interacts with coregulator proteins to modulate NR transcriptional activity.^{66–69} SRC1 and TIF2 are members of the steroid NR p160 coactivator family,^{70,71} and similar to TIF2,

increased expression of SRC1 has been associated with recurrent PCa.^{13,70–73} Based on the activity profiles of the test compounds in the AR-TIF2 PPIB assay, we anticipated that the three AR antagonists and 17-AAG would also be active in the two M2H assays. It was surprising therefore that all seven test compounds inhibited DHT-induced AR-LBD interactions with the TIF2 and SRC1 coactivators in a concentration-dependent manner (*Figs. 1–3* and *Table 2*). However, abiraterone acetate, compound No. 10, and EPI-001 also displayed substantial concentration-dependent cytotoxicity in HEK 293 cells, which may have contributed to their apparent activity in the M2H assays. None of the test compounds were selective in their ability to disrupt DHT-induced AR-LBD M2H interactions with either TIF2 or SRC1. We anticipated that AR-TIF2 PPI inhibitors/disruptors would effectively block DHT-induced transactivation, and indeed, only the three AR antagonists and 17-AAG inhibited DHT-induced AR-FL-GFP PSA6.1-luciferase reporter activity in a concentration-dependent manner (*Fig. 4* and *Table 2*). As expected, the anti-Cyp17a1 inhibitor abiraterone acetate did not inhibit DHT-induced AR-FL-GFP PSA6.1-luciferase reporter activity. The two AR-NTD-targeted compounds, compound No. 10 and EPI-001, also did not inhibit ligand-activated AR-FL transcription. Reassuringly, the three antiandrogen AR antagonists flutamide, bicalutamide, and enzalutamide competitively displaced H^3 -DHT binding to AR-LBD, and produced calculable IC_{50} s in all the AF-2 surface assays that utilized DHT to induce a response (*Fig. 5* and *Table 2*). As anticipated, the Hsp90 inhibitor 17-AAG, the Cyp17a1 inhibitor abiraterone acetate, and the two AR-NTD-targeted compounds did not inhibit H^3 -DHT binding to the AR-LBD, nor were they active in any of the AF-2-focused assays, except for the M2H assays as discussed above (*Fig. 5* and *Table 2*). Our original intent had been to use the AR-LBD H^3 -DHT binding assay to identify and deprioritize AR-TIF2 PPI inhibitor/disruptor hits that were AR antagonists, in part, not only because of the number of existing FDA-approved PCa drugs with this mechanism of action but also because the inevitable development of drug resistance to such drugs limits the duration of their efficacy for CRPC.^{5,11,13} However, it is possible that a compound, which binds to an allosteric binding site to induce a conformational change, that blocks AR-TIF2 PPIs might also have a negative impact on orthosteric ligand binding.⁷⁴ Indeed, an allosteric modulator that blocked AR-TIF2 PPIs and ligand binding would have a desirable bioactivity profile. The AF-2 surface interacts with coactivators containing binding motifs encoded by LXXLL and/or FXXLF sequences to regulate androgen-dependent AR transactivation.^{14,37–39} Based on their established targets

and mechanisms of action, we did not anticipate that any of the test compounds would block TIF2 Box III LXXLL peptide binding to the AR-LBD (Fig. 6 and Table 2). However, the Hsp90 inhibitor 17-AAG produced a calculable IC₅₀ and flutamide displayed evidence of concentration-dependent inhibition of TIF2-LXXLL-peptide binding to the AR-LBD. We used the SwissADME program hosted by the Swiss Institute of Bioinformatics to evaluate whether any of our test compounds might be pan-assay interference compounds (PAINS)⁷⁵ that interfere with the ALPHA Screen assay format. Only 17-AAG was flagged as a potential PAINS due to the presence of a quinone within the structure. Interestingly, only the Hsp90 inhibitor 17-AAG displayed the desired bioactivity profile of an AR-TIF2 PPI inhibitor/disruptor; it inhibited the AR-TIF2 PPIB assay and produced calculable IC₅₀s in all the AF-2 surface assays, but not an AR antagonist. However, since 17-AAG is an Hsp90 ATPase inhibitor, its activity in the AR-TIF2 PPIB and AF-2-focused assays was likely due to its ability to block the Hsp90-mediated maturation, by folding and maintenance of AR in a high-affinity conformation for ligand binding, and/or by blocking AR nuclear trafficking.

The AF-1-focused assays included a ligand-independent AR-NTD transactivation assay conducted in HEK 293 cells, and the PSA6.1 and UBE2C promoter-driven luciferase reporter assays conducted in PC3-AR-V7-GFP cells. Unexpectedly, we observed that the pGal4-AR-NTD construct did not respond to either SRC1 or TIF2 coactivation (Supplementary Fig. S6B), which was inconsistent with the published reports.^{14,15,34,42,76,77} There were, however, some differences in how our assays were configured compared to those other studies. The other groups conducted their assays in CV-1 cells, and the M2H bait and prey constructs were flipped with the TIF2 and SRC1 coactivators being inserted into the Gal4-DBD plasmids and AR-NTD inserted into the VP16 vector. We, therefore, used our assay to measure constitutive AR-NTD-mediated transactivation. Surprisingly, all seven test compounds inhibited constitutive AR-NTD transactivation in a concentration-dependent manner (Fig. 7 and Table 3). Once again, however, abiraterone acetate, compound No. 10, and EPI-001 also displayed substantial concentration-dependent cytotoxicity in HEK 293 cells, which may have contributed to their apparent activity in the AR-NTD transactivation assay. In the two reporter assays conducted in PC3-AR-V7-GFP cells, only four compounds produced calculable IC₅₀s: compound No. 10, EPI-001, 17-AAG, and flutamide (Figs. 8 and 9, and Table 3). It was reassuring that compound No. 10 and EPI-001, which target the AR-NTD, inhibited AR-V7-induced reporter activity. It is possible that the Hsp90 molecular chaperone

inhibitor 17-AAG was active in both reporter assays because of its ability to inhibit the maturation, folding, and activity of the luciferase enzyme; however, the ability of the AR antagonist flutamide to inhibit AR-V7-driven reporter responses was unexpected.

PCa progression to CRPC requires continued AR signaling even after treatment with ADTs. Overexpression of AR coactivators and the emergence of AR splice variants both promote continued transcriptional activation under androgen-depleted conditions, which constitute drug resistance mechanisms contributing to CRPC progression. We have prosecuted an HCS campaign to identify hit compounds that can inhibit or disrupt the PPIs between AR and TIF2, one of the coactivators implicated in CRPC disease progression.^{22–}

²⁵ Since an ideal inhibitor/disruptor of AR coactivator PPIs would target both the AF-2 and AF-1 domains, which serve as binding surfaces for coactivators, we have developed and validated a panel of assays to interrogate and prioritize hits that disrupt both transactivation surfaces (article in preparation). Five AF-2- and three AF-1-focused assays were developed. The TIF2 and SRC1 M2H assays will be used to confirm that AR-TIF2 PPIB hits block AR AF-2 coactivator interactions and to evaluate p160 coactivator selectivity. The PSA6.1-Luc reporter assay conducted in PC3-AR-GFP cells will confirm that hits block ligand-dependent AR-FL transcriptional activation. The AR-LBD H³-DHT binding assay will enable us to identify hits that are AR antagonists, or that may be negative allosteric modulators that also affect ligand binding. The AR-LBD TIF2 Box III LXXLL peptide binding assay will enable us to identify hits that inhibit/disrupt an established mechanism of coactivator binding to the AF-2 surface. The constitutive AR-NTD transactivation assay will allow us to test whether hits can also inhibit ligand-independent AF-1-mediated transcriptional activation, and the PSA6.1 and UBE2C reporter assays conducted in PC3-AR-V7-GFP cells will be used to determine if hits can also block ligand-independent splice variant transcriptional activation. AR-TIF2 PPI inhibitor/disruptor hits that can inhibit coactivator recruitment and AR transcriptional activation at both the AF-2 and AF-1 surfaces would be prioritized for lead optimization and have the potential to be developed into novel drug candidates for CRPC therapy.

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DISCLOSURE STATEMENT

The authors declare no conflicts of interest.

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SUPPLEMENTARY MATERIAL

Supplementary Figure S1
Supplementary Figure S2
Supplementary Figure S3
Supplementary Figure S4
Supplementary Figure S5
Supplementary Figure S6
Supplementary Figure S7
Supplementary Table S1
Supplementary Table S2

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Abbreviations Used

% CVs = percent coefficient of variance
 17-AAG = 17-allylaminogeldanamycin
 ADT = androgen deprivation therapy
 AF-1 = activation function 1
 AF-2 = activation function 2
 AR = androgen receptor
 AR-NTD = N-terminal domain of AR
 ARE = androgen response element
 AR-V7 = androgen receptor splice variant 7
 CPMs = counts per minute
 CRPC = castration-resistant prostate cancer
 DBD = DNA-binding domain
 DHT = dihydrotestosterone

DMEM/F12 = Dulbecco's modified Eagle medium: nutrient mixture F-12

DMSO = dimethyl sulfoxide

FBS = fetal bovine serum

FL = full length

Gal4 = galactose 4 DNA binding domain

GFP = green fluorescent protein

H³-DHT = tritiated dihydrotestosterone

HCS = high-content screening

HEK 293 = human embryonic kidney 293 cells

Hsp = heat shock protein

IC₅₀ = 50% inhibition concentration

IXM = ImageXpress Micro

LBD = ligand-binding domain

Luc = luciferase

LXXLL = coactivator leucine-rich binding motifs

M2H = mammalian 2-hybrid

NCI = National Cancer Institute

NID = nuclear interaction domain

NR = nuclear receptor

PAINS = pan-assay interference compounds

PBS = phosphate-buffered saline

PCa = prostate cancer

PCR = polymerase chain reaction

pGal4 = DNA-binding domain of the Gal4 protein
 derived from yeast

PI = principal investigator

PPI = protein-protein interaction

PPIB = protein-protein interaction biosensor

PSA = prostate-specific antigen

RFP = red fluorescent protein

RLUs = relative light units

RT = room temperature

S:B = signal-to-background

SA-DB = streptavidin donor beads

SFM = serum-free media

SRC = steroid receptor coactivator

TAU5 = transactivation unit 5

TE = translocation enhanced

TIF2 = transcriptional intermediary factor 2

U-2 OS = U-2 osteosarcoma cell line

UBE2C = ubiquitin-conjugating enzyme E2C