Published in final edited form as:

Mol Cancer Ther. 2020 January ; 19(1): 75–88. doi:10.1158/1535-7163.MCT-19-0489.

A novel small molecule targets androgen receptor and its splice variants in castration-resistant prostate cancer

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Abstract

Reactivation of AR appears to be the major mechanism driving the resistance of CRPC to second generation antiandrogens and involves AR overexpression, AR mutation, and/or expression of AR splice variants lacking LBD. There is a need for novel small molecules targeting AR, particularly those also targeting AR splice variants such as ARv7. A high-throughput-high-content screen was

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Author contributions

Z.Y. performed cell line experiments, RNA-Seq analysis and animal studies, analyzed data and wrote the manuscript. D.W. performed pull down assay, radioactive ligand binding assay, CHIP assay, and part of PCR assay and luciferase reporter assay and helped write the manuscript. J.K.J., K.T. and T.M. generated, synthesized and analyzed the JJ-450 analog. L.E.P. performed animal studies, analyzed data and helped write the manuscript. R.A. and A.B.C. analyzed RNA-Seq data. J.Z., W.C. and M.Z. performed luciferase assays, and assisted with also animal studies. Q.S. performed luciferase assays. H.D. and Z.W. assisted with cell line and animal studies. U.C. supervised the analysis of RNA-Seq data and assisted with manuscript preparation. J.B.N. helped with the experimental design, data analysis and manuscript preparation. P.W. supervised the synthesis and analysis of JJ-450 and analogs, experimental design, data analysis and manuscript preparation. Z.W. conceived and supervised the study and designed experiments, analyzed data and manuscript preparation.

Conflict of interest: Z.W., J.B.N., J.K.J. and P.W. are part of a patent involving these small molecules. The US Patent # 9,708,276 Small Molecules Targeting Androgen Receptor Nuclear Localization and/or Level in Prostate Cancer and Patent Application PCT/US2014/056369 Compounds for Treating Prostate Cancer were created through the office of technology management at the University of Pittsburgh. The patent is optioned by UPMC Enterprises. The remaining authors have no competing interests.

previously reported that led to the discovery of a novel lead compound, 2-(((3,5-dimethylisoxazol-4-yl)methyl)thio)-1-(4-(2,3-dimethylphenyl)piperazin-1-yl)ethan-1-one (IMTPPE), capable of inhibiting nuclear AR level and activity in CRPC cells, including those resistant to enzalutamide. A novel analog of IMTPPE, JJ-450, has been investigated with evidence for its direct and specific inhibition of AR transcriptional activity via a pulldown assay and RNA-seq analysis, PSA-based luciferase, qPCR, and ChIP assays and xenograft tumor model 22Rv1. JJ-450 blocks AR recruitment to androgen responsive elements (AREs) and suppresses AR target gene expression. JJ-450 also inhibits ARv7 transcriptional activity and its target gene expression. Importantly, JJ-450 suppresses the growth of CRPC tumor xenografts, including ARv7-expressing 22Rv1. Collectively, these findings suggest JJ-450 represents a new class of AR antagonists with therapeutic potential for CRPC, including those resistant to enzalutamide.

Introduction

Prostate cancer is the most commonly diagnosed non-cutaneous malignancy and the second leading cause of cancer death among men in the United States (1). Androgen deprivation therapy (ADT) is the standard and initially effective treatment for patients with metastatic prostate cancer, but most will eventually develop castration-resistant prostate cancer (CRPC), defined as androgen receptor (AR) activation despite castrate levels of androgen. (2-6). Second generation AR inhibitors were developed or are under development for the treatment of CRPC, including the selective androgen biosynthesis inhibitor abiraterone (7), and AR antagonists, such as enzalutamide (MDV3100) (8), apalutamide (ARN-509) (9,10), and darolutamide (ODM-201) (11). These inhibitors can prolong the survival of CRPC patients for several months with most developing resistance (12-14).

Resistance to second generation AR inhibitors is largely the result of AR reactivation through multiple mechanisms, including up-regulation of AR and CYP17 expression, emergence of AR point mutations, constitutively active AR splice variants, and increased intratumoral androgen synthesis (15-18). As such, AR remains an important therapeutic target for CRPC resistant to second generation AR inhibitors, with the need for novel anti-AR agents.

The current group of FDA-approved AR targeting agents act either directly or indirectly through the AR ligand-binding domain (LBD), which increases the likelihood of cross-resistance to these therapeutic agents. To overcome resistance to enzalutamide and/or abiraterone, new anti-AR therapeutics targeting different domain(s) of AR, such as the N-terminal domain (NTD) or DNA-binding domain (DBD) is a reasonable strategy. Several small molecules, including EPI-001 for targeting NTD (19), VPC-14228 and VPC-14449 for targeting DBD (20), and SARDs for targeting both DBD and LBD (21), are being developed for CRPC treatment. The identification and development of small molecules targeting AR through other mechanisms will enhance the possibility of clinically useful AR inhibitors.

Recently, a high-throughput/high-content screening campaign of 219,055 small molecules was reported, identifying a novel AR antagonist, 2-(((3,5-dimethylisoxazol-4-yl)methyl)thio)-1-(4-(2,3-dimethylphenyl)piperazin-1-yl)ethan-1-one (IMTPPE), based on its ability to inhibit nuclear AR levels in CRPC cells (22). IMTPPE blocked the expression

of AR-target genes, inhibited the proliferation of AR-positive cancer cells, and suppressed the growth of xenograft tumors expressing AR variants. IMTPPE inhibition of AR did not require the LBD, as this compound inhibited the activity of a mutant AR lacking the LBD (23). Since the structure of IMTPPE is distinct from known anti-androgens and it inhibited AR lacking the LBD, IMTPPE can be considered as a new AR antagonist and may provide an opportunity to inhibit growth in CRPC resistant to second generation AR inhibitors.

Here, the mechanism of IMTPPE's inhibition of AR is further investigated with characterization of JJ-450, an IMTPPE scaffold variant with improved potency and physicochemical properties. JJ-450 (originally designated Compound 27) is a racemic mixture of 2 stereoisomers, (–)-JJ-450 and (+)-JJ-450, with (–)-JJ-450 being ~9 fold more potent than (+)-JJ-450 in the luciferase PSA reporter assay (24). In this study, the binding of IMTPPE to AR was tested, JJ-450's inhibition of AR and ARv7 transcriptional activities was assessed, JJ-450's specificity of AR inhibition was compared to enzalutamide, and the efficacy of JJ-450 in prostate xenograft tumors was determined, including those resistant to enzalutamide.

Methods

Cell lines and key resources

LNCaP, 22Rv1, VCaP and PC3 prostate cancer cell lines were obtained from the American Type Culture Collection (ATCC). C4-2 (25), LAPC4 (26), CWR-R1 (27) and GFP-ARv7:PC3 (28) cell lines were kindly provided by Dr. Leland WK Chung (Cedars-Sinai Medical Center), Dr. Robert Reiter (UCLA), Dr. Christopher W Gregory (UNC), and Dr. Michael Mancini (BCM), respectively. C4-2-PSA-rl (24) and PC3-AR (29) stable cell lines were generated internally. All cells were maintained at 37°C in a 5% CO₂ incubator, grown in complete medium supplemented with 10% FBS (Atlanta Biologicals), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 µg/ml). LNCaP, 22Rv1, PC3, C4-2 and CWR-R1 cells were grown in complete RPMI medium (Corning). VCaP cells were maintained in complete DMEM medium (Lonza). LAPC4 cells were grown in IMDM (HyClone) supplemented with 1 nM R1881 (PerkinElmer). PC3-AR cells were maintained in complete RPMI medium supplemented with 500 µg/mL G418. GFP-ARv7:PC3 cells were grown in complete DMEM/F12 (Corning) supplemented with 500 µg/mL G418. C4-2-PSA-rl cells were cultured in RPMI supplemented with 10 µg/ml puromycin and 500 µg/mL G418. The genetic identity of 22Rv1, C4-2, VCaP, and LNCaP cell lines was authenticated in 2016 using DNA fingerprinting by examining microsatellite loci in a multiplex PCR (AmpFISTR Identifier PCR Amplification Kit, Applied Biosystems, Foster City, CA) by the University of Pittsburgh Cell Culture and Cytogenetics Facility. Cell lines were confirmed as mycoplasma free by PCR testing on a monthly basis and all key experiments were performed using cell lines between passages 4 to 28. Key resources are listed in Supplementary Table S1.

Xenograft Mouse Models

Male mice aged 6-8 weeks-old Crl:SHO-*Prkdc^{scid}Hr^{hr}* were obtained from Charles River Laboratory (Washington, MA). Animal care and use were reviewed and approved by the

Institutional Animal Care and Use Committee (IACUC) of the University of Pittsburgh (protocol #17019973). LNCaP (3×10^6 cells), C4-2 (3×10^6 cells), VCaP (2.7×10^6 cells) or 22Rv1 (1×10^6 cells) suspended in 150 μ L medium were gently mixed with 150 μ L of Matrigel (Corning, NY) and then inoculated subcutaneously in the right flank region of male Crl:SHO-Prkdc^{scid}Hr^{hr} mice. Tumor volume was calculated using the modified ellipsoid formula: length $\times$ width² $\times$ 0.52 (30). For the short-term study in the LNCaP model (Figure 6A-E), castration was performed when tumor volume reached ~ 200 mm³. When tumor volume reached ~ 300 mm³, mice were randomized and treated with enzalutamide (i.p. 10 mg/kg body weight) or JJ-450 (i.p. 25 mg/kg body weight) every day for a total of seven injections. Tumor volume was measured with calipers and blood was collected from the saphenous vein before the initial injection and the fourth injection. Mice were euthanized four hours after the last injection. For the long-term study in the LNCaP model (Figure 6F&G), castration was performed when tumor diameter reached 7-8 mm. Mice were randomized and treated with JJ-450 (o.g. 10 mg/kg body weight, i.p. 10 mg/kg body weight, or o.g. 75 mg/kg body weight) six days on, one day off, initiated 10-22 days after castration. Tumor volume was measured twice a week. For the 22Rv1 model (Figure 7A, B, & C), castration was performed when tumor diameter reached 5 mm. Mice were randomized and treated with JJ-450 (i.p. 10 mg/kg body weight, or i.p. 75 mg/kg body weight) every day, initiated at the time of castration. Tumor volume was measured three times a week. For the VCaP model (Figure 7D), castration was performed when tumor volume reached ~ 250 mm³. Mice were randomized and treated with JJ-450 (i.p. 75 mg/kg body weight) or enzalutamide (i.p. 10 mg/kg body weight) every day, initiated two weeks after castration. Animals displaying any signs of morbidity or ulceration of tumors were euthanized and removed from the study. Tumor volume was measured three times a week.

Trans-scrotal castration was performed under isoflurane anesthesia with proper aseptic and antiseptic technique (31). Mice were weighed weekly using a digital scale or a triple-beam balance, and blood was collected via saphenous vein or by cardiac puncture at euthanasia and tumors were preserved for further biochemical and immunofluorescence analysis. Survival analysis was carried out using a tumor volume of 2000 mm³ as a surrogate for mortality and analyzed using GraphPad Prism 6.0 software (GraphPad Software). Statistical significance for Kaplan-Meier analysis was determined using the log-rank (Mantel-Cox) test.

Small molecule inhibitors

Small molecule analogs of IMTPPE were synthesized as described previously (24). One active analogue (**325**) was conjugated to agarose (32) to generate beads (**403**) suitable for an AR pulldown assay. Two capped agarose beads (**403c**, **974**) were used as controls. The structures of these beads were provided in Figure 1A. The chiral resolution of JJ-450 to give (–)-JJ-450 and (+)-JJ-450 was performed by chiral SCF chromatography. Doses were based on solubility data established for IMTPPE previously (23,24).

AR pulldown assay

C4-2 cells cultured in complete RPMI medium in 15 cm dishes were used directly or transfected with desired plasmids for 48 h prior to use for nuclear extract preparation. Cells

were trypsinized and cell pellets were resuspended with 1 ml TEDG buffer (1.5 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM sodium molybdate, 1 mM DTT, 10% glycerol) with protease inhibitors. To collect nuclear extracts, cells were homogenized using a glass Dounce tissue homogenizer and centrifuged to pellet cell nuclei. Cell nuclei were washed and resuspended with TEDG buffer, followed by sonication. To collect whole cell extracts, cells were sonicated directly followed by centrifugation. Supernatant was transferred to a fresh tube. Control beads (30 μ L) were used to preclear 500 μ L lysates or purified AR in TEDG buffer. Beads were blocked using 2.5% BSA in PBS. Precleared cell lysates were diluted with TEDG buffer with protease inhibitor and incubated with beads at 4 °C overnight. Beads were collected and washed with buffer (175 mM NaCl, 20 mM Tris-HCl, pH7.5, 1% NP-40, 15% Glycerol, 1.5 mM MgCl₂, 2 mM EDTA) with protease inhibitors three times. Proteins were eluted with elution buffer (50 mM NaHCO₃ and 1% SDS) at 4 °C and denatured with SDS loading buffer, following immunoblotting analysis.

Radioactive ligand binding assay

Cytoplasmic extracts were prepared using TEDG buffer for radioactive ligand binding assay. The extracts were incubated with indicated concentrations of ³H-DHT in the presence or absence of 10 μ M IMTPPE at 4°C overnight. Dextran coated charcoal or Sephadex G25 columns were used to remove free ligand. ³H-DHT in supernatant was measured using a scintillation counter. Percentage of ³H-DHT bound to AR in lysate was calculated.

Plasmids and transfection

The expression vector pEGFP-C1 (Clontech) was used to generate GFP-AR or GFP-NAR (AR lacking LBD) as described previously (33). pEGFP-C1-ER α was kindly provided by Dr. Gerard Evan (34). Renilla luciferase reporter (pRL-TK) was purchased from Promega (Madison, WI). PSA luciferase reporter vector (pPSA6.1-Luc) was a kind gift provided by Dr. Marianne Sadar (35). Glucocorticoid receptor (GR) expression vector and MMTV-luciferase reporter were from Dr. Donald DeFranco (36). The 3X ERE TATA luc was a gift from Donald McDonnell (Addgene plasmid # 11354). pEGFP-C1-AR V7 was a gift from Michael Mancini & Marco Marcelli (Addgene plasmid # 86856). Plasmids were double CsCl gradient purified for transient transfection. Cell transfection was performed with PolyJet DNA In Vitro Transfection Reagent (SigmaGen Laboratories) according to manufacturer's protocols.

Western blot analysis

Cell lysates were prepared in RIPA buffer containing protease inhibitor cocktails (Sigma-Aldrich). Protein extract was boiled in SDS sample buffer, size fractionated by SDS-PAGE, and transferred onto a PVDF membrane (BioRad). Membranes were blocked for 1 hour at room temperature in TBST buffer containing 5% nonfat milk, then incubated with the following primary antibodies overnight: anti-AR rabbit polyclonal (1:1000, Santa Cruz, sc-816), anti-PSA goat polyclonal (1:500, Santa Cruz, sc-7638), anti-GFP rabbit polyclonal (1:1000, Santa Cruz, SC-8334), anti-UBE2C rabbit polyclonal (1:1000, CST, 14234S) and anti-GAPDH mouse monoclonal (1:10000, Santa Cruz, sc-47724 HRP). After three washes in TBST, membranes were incubated with secondary antibody diluted in blocking buffer for 1 hour at room temperature. Following three washes in TBST, Enhanced Chemiluminescent

(ECL) was performed using ECL kit (BioRad) and signals detected using ChemiDoc Imaging System (Bio-Rad).

Quantitative real-time PCR assays

To isolate RNA from cells, the RNeasy Mini kit (Qiagen) was used following the manufacturer's protocol. To isolate RNA from xenograft tumors, tumor samples stored in RNAlater-ICE (ThermoFisher) were homogenized in 600 μ L lysis buffer using a digital ULTRA-TURRAX homogenizer (IKA-labortechnik). Samples were then centrifuged at 13000 rpm/min for 3 min. The supernatant was mixed with equal volume of 70% ethanol, and the RNA was further purified using the RNeasy Mini kit (Qiagen). RNA was reverse transcribed using the PrimeScript RT Reagent Kit (TaKaRa). All reactions were performed in triplicated on an ABI StepOnePlus™ system (Applied Biosystems) using SYBR Advantage qPCR Premix (TaKaRa). The relative quantity of the target genes was calculated using the $\Delta\Delta$ Ct method by comparing average Ct of the target and mean Ct of the housekeeping gene, GAPDH. Primer sequences are provided in Supplementary Table S1.

RNA-sequencing analysis

LNCaP cells were plated in 6-well plates with 5% charcoal-stripped FBS (cFBS) in phenol red-free RPMI medium (Corning) for 24 h, then treated with ethanol or 1 nM R1881 and DMSO, IMTPPE (10 μ M), (-)-JJ-450 (10 μ M), enzalutamide (10 μ M), or (+)-JJ-450 (25 μ M) for an additional 24 h in triplicate prior to total RNA isolation using an RNeasy Mini kit (Qiagen). RNA-Seq library construction and sequencing were performed by the Genomics Research Core of University of Pittsburgh. Libraries were prepared using the TruSeq Stranded mRNA kit (Illumina) following the manufacturer's protocol. Briefly, starting from 1 μ g total RNA, mRNA isolated from LNCaP cells was purified using oligo-dT magnetic beads. Following two rounds of purification, mRNA was fragmented. First strand reverse transcription was performed with random hexamer primers. After second strand synthesis, blunt ended cDNA fragments were A-tailed followed by ligation of indexed sequencing adapters. PCR amplification provided selective enrichment of DNA with adapters ligated to both ends and was followed by library quantity and quality assessment using fluorometric assay (Qubit) and Agilent DNA 1000 TapeStation assay, respectively. Final libraries were normalized to 2 nM, pooled and diluted for instrument loading. Flowcells for the NextSeq 500 were seeded with 1.8 pM denatured library for automated cluster formation and 2 $\times$ 75 paired end sequencing. Sequencing generated between 42,985,974-56,182,970 pairs of reads for the 18 sequenced samples. FASTQC was run to examine the quality of the reads. Cutadapt v1.8.3 software was used for adapter trimming and quality trimming at a setting of 20 (37). The True Seq (Illumina) adapter sequences used for adapter trimming were AGATCGGAAGAGCACACGTCTGAACTCCAGTCA (left reads) and AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT (right reads). Data analyses were performed by the Cancer Bioinformatics Services (CBS), a shared resource for UPMC Hillman researchers. Briefly, each sample was mapped to the Human Ensembl reference genome GRCh38 using the fast alignment tool HISAT2 v2.1.0 with default settings and library type set to RF (38). Transcripts in GRCh38 were quantified using HTSeq v0.9.0 using default settings for paired reads and library type set to reverse (39). An R Bioconductor package, edgeR, was used for differential gene analysis comparing drug

treatment groups with a gene expression filter of > 1 CPM counts in at least 3 samples as expression above background (40). Heatmaps visualizing and comparing mRNA expression were generated using R.

Luciferase reporter assays

C4-2 or 22Rv1 cells were co-transfected with appropriate expression vectors and/or indicated report vectors in 5% cFBS, phenol red-free RPMI medium prior to treatment for 24 hours. GFP-ARv7:PC3 cells were co-transfected with pRL-TK and pPSA6.1-Luc report vectors in 5% cFBS, phenol red-free RPMI medium for 14 hours before treatment. After treatment, luciferase assays were performed as described previously (23). Briefly, cells were lysed and luciferase activity was assayed using a Dual-Luciferase Reporter Assay kit (Promega) and measured using LMax II Microplate Reader (Molecular Devices) following the manufacturer's recommendations. The firefly luciferase activity was normalized to Renilla luciferase activity.

PSA ELISA assay

C4-2 cell culture medium supernatant was collected by centrifugation at 1000 g for 15 min at 4 °C. Mouse serum was prepared by allowing blood samples to clot for 30 minutes at room temperature, followed by centrifugation at 1000 g for 15 min at 4 °C. Culture medium supernatant and serum samples were stored at -80 °C before ELISA assay. Total PSA level was measured using a Human Kallikrein 3/PSA Quantikine ELISA Kit (R&D) according to the manufacturer's protocols.

Chromatin immunoprecipitation (ChIP)

C4-2 cells were cultured in 5% cFBS, phenol red-free RPMI medium for 2 days, then treated with vehicle (ethanol) or 1 nM R1881, combined with DMSO, 10 μ M IMTPPE, or JJ-450 for 2 h. Cells were cross-linked using 1% paraformaldehyde for 10 minutes on a shaker at room temperature, and 0.125 M glycine was used to stop crosslinking. After cross-linking, cells were washed and collected by centrifugation with cold PBS at 4°C. Cell pellets were lysed with cell lysis buffer (5 mM PIPES, pH8.0, 85 mM KCl, and 0.5% NP40) plus 100X PMSF and 100X PIC on ice for 10 min and cell nuclei were pellet with centrifugation at 5000 rpm for 5 min. Nuclei pellets were resuspended with nuclei lysis buffer (50 mM Tris-Cl, pH 8.0, 10mM EDTA, and 1%SDS) plus 100X PMSF and 100X PIC. Sonication at desired intensity was used to shear chromatin to 200 bp-1000 bp fragments on ice. After preclearing with 20 μ L salmon sperm DNA/Protein A agarose (EMD Millipore) at 4 °C for 1 h, the lysates were diluted in 10-fold with IP dilution buffer (0.01% SDS, 1.1% TritonX-100, 1.2 mM EDTA, 16.7 mM Tris-Cl, pH8.1, and 167 mM NaCl) and 50 μ L diluted samples were saved as inputs. Anti-AR or rabbit IgG antibodies (4 μ g) were added to each sample and incubated on a rotating platform at 4 °C overnight. On the following day, 40 μ L salmon sperm DNA/Protein A agarose was added for 2 h. Agarose beads were washed with dialysis buffer (2 mM EDTA, 50 mM Tris-Cl, pH 8.0, and 0.2% Sarkosyl) 2 times and wash buffer (100 mM Tris-Cl, pH 9.0, 500 mM LiCl, 1% NP40 and 1% DOC) 4 times. The protein/DNA complexes were eluted with 50 mM NaHCO₃ and 1% SDS at room temperature. All samples and inputs were incubated with 0.2 M NaCl at 67 °C overnight and then treated with RNase A and proteinase K. DNA was purified using QIAquick PCR purification kit

(Qiagen) and amplified using SYBR Green PCR mix (ThermoFisher) on an ABI StepOnePlus™ system (Applied Biosystems). Input chromatin was used for estimation of relative enrichment. Primer sequences for AREs at regulatory regions of PSA and TMPRSS2 genes are provided in Supplementary Table S1.

Immunohistochemistry of xenografts

Immunohistochemical staining for Ki67 was performed on LNCaP tumors subjected to short term treatment using a standard protocol (41). Formalin-fixed paraffin-embedded tissue sections were de-paraffinized and antigen retrieval was performed using antigen unmasking solution (Vector Laboratories) at 98-100 °C for 20 min. Endogenous peroxidase was inactivated by incubation with 1% hydrogen peroxide for 30 min at room temperature. Sections were then incubated sequentially in 1.5% blocking serum for 1 h at room temperature, primary antibody (anti-Ki67 rabbit polyclonal (1:200, Abcam, ab15580), anti-cleaved caspase 3 rabbit monoclonal (1:100, CST, 9664S), or anti-AR rabbit monoclonal (1:100, CST, 5153S)) diluted in 1.5% serum at 4 °C overnight, followed by incubation with biotinylated secondary anti-rabbit antibody (Santa Cruz) for 1 h, AB enzyme reagent (avidin and biotinylated HRP) (Santa Cruz) for 30 min at room temperature, followed by a 2-5 min colorimetric development with peroxidase substrate kit (Vector Laboratories). Slides were counterstained with hematoxylin and mounted. Images of immunostained sections were acquired using a Leica DMLB microscope (Leica Microsystems Inc). Ki67 positive cell density was determined by analysis of sections from at least 8 different xenografts from each group. Proliferative index was determined from 5 fields per tumor imaged at 20x magnification with no overlap, Ki67 positive cells were counted to determine the average number of proliferating cells for each field. Positive cells were counted using Image-Pro Plus software (Media Cybernetics).

Mice randomization and compounds administration

The randomization method used for each animal study was based on the tumor volume or diameter from fast to slow tumor growth: 1; 2; 3; 4; 4; 3; 2; 1; 1... etc. All compounds were dissolved in DMSO and then diluted with EtOH and Kolliphor-PBS with the ratio 1:1:8 and administered to mice by i.p. injection or oral gavage.

Quantification and statistical analysis

Data are expressed as mean $\pm$ SD. Statistical significance, p value < 0.05, was determined by two-tailed Student's t-test or Log-rank Mantel-Cox Test using GraphPad Prism 6.0 software (GraphPad Software). The RNA-seq data in this paper are deposited at Gene Expression Omnibus under accession number GSE115395.

Results

IMTPPE directly binds to AR

IMTPPE was identified as a novel small molecule capable of inhibiting the nuclear localization and activity of AR in CRPC cells (22,24). It was not clear, however, if IMTPPE exerts its effects by direct binding to AR. To test if the IMTPPE chemotype interacts directly with AR, small molecule-conjugated agarose beads (**403**) were developed for AR pulldown

experiments, with an IMTPPE analog and a linker suitable for an agarose attachment (Figure 1A). The EC₅₀ of **26b** in the PSA luciferase assay is 7.9±2.8 μM, and the propargyl alcohol analog **325** has an EC₅₀ of 2.8±1.0 (22). The linker containing analog **404**, without conjugation to agarose beads, retained the ability to inhibit PSA-luciferase activity (Supplementary Figure S1). Control beads included **403c** agarose beads and **974**-conjugated agarose beads (Figure 1A). Transfected GFP-AR, but not GFP-Rb1, was pulled down by **403** beads, an effect that could be antagonized by IMTPPE (Figure 1B). Similarly, endogenous AR in C4-2 cells was pulled down by **403** beads, but not by **403c** control beads, and IMTPPE could compete with the pulldown of AR by **403** beads (Figure 1C). Furthermore, the **403** beads pulled down purified AR protein much more efficiently than #403c, which weakly pulled down the AR protein presumably due to non-specific binding (Figure 1D). Similar experiments to determine if JJ-450 could bind directly to AR were unsuccessful due to the low solubility of JJ-450. These results suggest that the IMTPPE scaffold could directly bind to AR.

Since IMTPPE could bind to AR directly, its effects on inhibiting androgen binding to AR were studied with radioactive ligand binding assays of IMTPPE on ³H-DHT binding to AR. In C4-2 cytoplasmic extracts, the percentage of bound ³H-DHT decreased as the ³H-DHT concentration increased from 0.1 nM to 10 nM, becoming excessive as compared to AR (Figure 1E). IMTPPE at 10 μM reduced low-dose (0.1 and 1 nM), but not high-dose (10 nM) ³H-DHT binding to AR in C4-2 cytoplasmic extracts (Figure 1E). IMTPPE had no effect on ³H-DHT binding to GFP-AR in PC3 cell extracts (Figure 1E). These observations suggest IMTPPE could inhibit ligand binding to endogenous AR in AR-positive C4-2 cells but not in transfected GFP-AR in AR-negative PC3 cells.

JJ-450 inhibits AR transactivation of and recruitment to target genes

The effects of JJ-450 with enzalutamide on AR transcriptional activity were studied using a PSA-luciferase assay (24). JJ-450 is racemic and can be resolved into (–)-(1*S*,2*R*)-JJ-450 and (+)-(1*R*,2*S*)-JJ-450 (Figure 2A) (24). Both (–)-JJ-450 and (+)-JJ-450 inhibited the PSA-luciferase activity in a dose-dependent fashion in C4-2-PSA-rl cells, with (–)-JJ-450 being more potent than (+)-JJ-450 (Figure 2B&2C). In the PSA-luciferase assays using 10 μM antagonists, enzalutamide was more effective at low concentrations of R1881 (0.1 nM), whereas (–)-JJ-450 was more potent at higher concentrations of R1881 (1 nM and 10 nM) (Figure 2C). This suggests that the mechanisms of AR inhibition by JJ-450 and enzalutamide are likely different.

The effects of JJ-450 on androgen induction of endogenous AR-target gene PSA at the protein level in LNCaP, C4-2, LAPC4 and 22Rv1 prostate cancer cells were also studied. Western blot analysis revealed that both (+)- and (–)-JJ-450 inhibited PSA protein levels, with (–)-JJ-450 being more potent in all the tested cell lines (Figure 2D). Both (+)- and (–)-JJ-450 also inhibited AR expression levels slightly at 25 μM (Figure 2D). JJ-450 also inhibited normalized secreted PSA levels in the culture medium in a dose-dependent manner (Supplementary Figure S2). Quantitative PCR analysis showed JJ-450 inhibition of R1881-induced mRNA expression of PSA (KLK3), EAF2 and NKX3.1, which are androgen responsive genes (42), in LNCaP (Supplementary Figure S3A), C4-2 (Supplementary Figure

S3B), VCaP (Supplementary Figure S3C), and 22Rv1 (Supplementary Figure S3D). However, the JJ-450 stereoisomers did not inhibit mRNA of either full-length AR (Supplementary Figure S4A) or ARv7 (Supplementary Figure S4B). These results suggest that JJ-450 could alter AR activity without reducing AR protein level.

To test if IMTPPE or its analogs could also inhibit AR recruitment to AREs *in vivo*, chromatin immunoprecipitation (ChIP) assay was performed using the C4-2 cell line as a model. As expected, the synthetic androgen R1881 significantly increased AR binding to AREs (Figure 2E). AR binding to AREs in the PSA gene enhancer (PSA-EN-ARE) and in the TMPRSS-2 gene (TMPRSS2-ARE) was significantly decreased by 10 μ M IMTPPE or its analog JJ-450, but not ARE in the PSA promoter (PSA-PR-ARE) (Figure 2E). AR mRNA and protein expression levels were not inhibited by JJ-450 at this concentration (Figure 2D and Supplementary Figure S4A). These results suggest IMTPPE and its analogs could block AR recruitment to AREs.

JJ-450 inhibits ARv7 transcriptional activity and target gene expression

The inhibition of PSA expression in ARv7-positive 22Rv1 cells by (+)- and (-)-JJ-450 suggested that these compounds may also inhibit ARv7 or any AR variants/mutants lacking LBD, in addition to inhibiting full-length AR. In 22Rv1 cells, enzalutamide and JJ-450 stereoisomers showed a dose-dependent inhibition of PSA-luciferase activity in the presence of R1881 (Figure 3A). JJ-450 stereoisomers, but not enzalutamide, inhibited PSA-luciferase activity in androgen-depleted conditions (Figure 3B), suggesting that JJ-450 stereoisomers could inhibit ARv7 activity.

To further test JJ-450 inhibition of ARv7 and mutant AR lacking LBD, C4-2 cells were co-transfected with luciferase reporters along with GFP, GFP-AR-V7 (28), or GFP-NAR (AR mutant lacking LBD) (33), followed by treatment with indicated compounds in androgen-depleted medium. In the absence of androgens, GFP-transfected C4-2 cells showed very low PSA-luciferase activity, whereas GFP-AR-V7 and GFP-NAR transfected C4-2 cells exhibited robust luciferase activity (Figure 3C). High concentrations of JJ-450 stereoisomers, but not enzalutamide, blocked PSA-luciferase induction by GFP-NAR or GFP-ARv7 (Figure 3C). As a control, Western blot analysis showed no obvious change in transfected GFP, or GFP-ARv7, GFP-NAR protein levels in C4-2 cells (Supplementary Figure S5A). Similarly, JJ-450 stereoisomers, but not enzalutamide, also inhibited PSA-luciferase induction by GFP-ARv7 in GFP-ARv7:PC3, a PC3 subline stably transfected with GFP-ARv7 (28) (Figure 3D). These results suggest that JJ-450 stereoisomers could target NTD and/or DBD domains in AR variants/mutants.

AR variant-mediated transcriptional programs appear to be different from ligand-induced AR-target genes (43,44), so the effect of JJ-450 on ARv7-target gene expression in 22Rv1 cells cultured in the absence of androgens was studied. The 22Rv1 cell line is resistant to enzalutamide, due in part to the expression of AR variants (45). The mRNA levels of ARv7 signature genes, UBE2C, BUB1B, CCNA2 and KIF15 (43), were inhibited by JJ-450 stereoisomers at 20 μ M, but not by enzalutamide (Figure 3E). Also, JJ-450 stereoisomers showed a dose-dependent inhibition of UBE2C protein expression, whereas enzalutamide showed minimal effect on UBE2C expression (Figure 3F). Similarly, UBE2C, BUB1B,

CCNA2 and KIF15 mRNA levels were also inhibited by JJ-450 stereoisomers in CWR-R1 cells under androgen-depleted condition (Supplementary Figure S5B).

JJ-450 inhibition of AR

In order to determine whether JJ-450 could modify the action of other steroid nuclear receptors, the effect of JJ-450 on the transcriptional activities of estrogen receptor (ER) and glucocorticoid receptor (GR) using luciferase reporter assays in C4-2 cells was examined. ER α transactivation of 3x ERE TATA luciferase reporter (46) in the presence of 1 nM 17 β -estradiol (E2) was unaffected by JJ-450 (Figure 4A). JJ-450 did not affect the protein levels of transfected GFP-ER α (Figure 4B). Similarly, GR transactivation of MMTV-luciferase reporter (36) in the presence of 50 nM dexamethasone was insensitive to JJ-450 (Figure 4C), and JJ-450 did not affect GFP-GR protein levels (Figure 4D). In C4-2 cells, 1 nM E2 and 50 nM dexamethasone were sufficient to induce robust ER α and GR transcriptional activity (Supplementary Figures S6A and S6B). These data suggest that JJ-450 could target AR specifically without affecting other steroid nuclear receptors.

To further evaluate the specificity of JJ-450 in the inhibition of AR function, RNA-seq analysis was performed with cultured LNCaP cells stimulated with R1881. In this study, LNCaP cells were cultured in androgen-depleted medium for 24 h and then treated for an additional 24 h with 1) vehicle, 2) 1 nM R1881, 3) 1 nM R1881 plus (–)-JJ-450, 4) 1 nM R1881 plus (+)-JJ-450, 5) 1 nM R1881 plus IMTPPE, and 6) 1 nM R1881 plus enzalutamide. Differential expression (DE) genes were defined as those genes showing absolute fold change (FC) ≥ 2 and false discovery rate (FDR) ≤ 0.05 . Multidimensional scaling (MDS) plots show the distinct cluster of samples with each treatment (Figure 5A). The transcriptome of LNCaP cells treated with R1881 plus (–)-JJ-450 was very similar to R1881 plus enzalutamide, and both were similar to the vehicle control. The transcriptome of LNCaP cells treated with R1881 plus IMTPPE and R1881 plus (+)-JJ-450 appeared to be similar to each other, but were different from the vehicle or the R1881 only group (Figure 5A). The gene expression profiles were also analyzed using unsupervised clustering and a heatmap was generated which included 13,490 DE genes. Consistent with the MDS analysis, a heatmap showed that most gene expression changes induced by R1881 were repressed by (–)-JJ-450 or enzalutamide (Figure 5B), while (+)-JJ-450 and IMTPPE could repress part of the R1881-induced gene expression program and induced differential expression of several/many additional genes (Figure 5B). Compared to R1881 plus enzalutamide, R1881 plus IMTPPE and R1881 plus (+)-JJ-450 induced 2856 and 2878 DE genes, respectively, (Figure 5C; Supplementary Table S2). There were 186 DE genes between R1881 plus enzalutamide and the vehicle control, and only 56 DE genes between R1881 plus (–)-JJ-450 and the vehicle control (Figure 5D; Supplementary Table S3). As anticipated, R1881 induced significant changes in transcripts previously identified as androgen-response genes. A heatmap generated for 25 androgen responsive genes also verified that our compounds inhibited the expression of AR-regulated genes, with (–)-JJ-450 having similar specificity as enzalutamide (Supplementary Figure S7). These data indicate (–)-JJ-450 could inhibit R1881 activation of AR completely, with specificity and potency similar to enzalutamide.

JJ-450 inhibition of AR in relapsed LNCaP xenograft prostate tumors

In xenograft tumor experiments, the racemic mixture of JJ-450 was used to reduce the number of animals and quantity of compound required. FDA-approved bicalutamide is currently used as a racemic mixture for prostate cancer treatment (47,48), therefore using a racemic mixture of JJ-450 in animal studies has potential clinical relevance. Mice bearing subcutaneous LNCaP tumor xenografts were castrated when tumor volume reached ~200 mm³. After tumors relapsed and reached a volume of ~300 mm³, mice were randomized and intraperitoneally (i.p.) injected with vehicle, enzalutamide (10 mg/kg body weight), or JJ-450 (25 mg/kg body weight) every day for a total of seven injections. Both enzalutamide and JJ-450 inhibited growth of relapsed LNCaP tumors, serum PSA level normalized to tumor volume, and tumor proliferation marker Ki67 as compared to vehicle (Figure 6A, 6B, 6C, & 6D). mRNAs of two AR-target genes, PSA and FKBP5, were also inhibited by J-450 and enzalutamide (Figure 6E). However, JJ-450 and enzalutamide did not affect caspase-3 immunostaining or the AR level and nuclear localization in relapsed LNCaP tumors (Figure 6C).

To further verify the effect on relapsed LNCaP xenografts, mice with subcutaneous LNCaP xenografts were castrated when tumor diameter reached 7-8 mm and treated long-term with JJ-450 at indicated dosages with either oral gavage (o.g.) or i.p. injection, six days on, one day off, started ten days after castration (Figure 6F). All the JJ-450 treatment regimens inhibited the LNCaP tumor growth as compared to the vehicle treated mice (Figure 6F). Median survival times treated with JJ-450 o.g 10 mg/kg body weight, i.p. 10 mg/kg body weight, and o.g. 75 mg/kg body weight were 47, 55, and 54 days, respectively, significantly longer than the median survival time (39 days) of vehicle group (Figure 6G). These results suggest that JJ-450 could block AR function *in vivo* and inhibit tumor growth of relapsed LNCaP tumors.

JJ-450 inhibition of CRPC xenograft tumors that express AR splice variants.

The effect of JJ-450 on CRPC tumors that express full-length AR and AR variants was evaluated using the 22Rv1 and VCaP xenograft tumor models (49-53). The 22Rv1 tumor volume in animals treated with JJ-450 was reduced by more than 60% compared to the vehicle treated group at both low (10 mg/kg body weight) and high (75 mg/kg body weight) doses (Figure 7A). Median survival time for animals treated with JJ-450 was 43 and 49 days, at low and high doses respectively, which was significantly longer than the median survival time of 25 days for the vehicle control groups (Figure 7B). Mice treated with JJ-450 showed no changes in body weight (Figure 7C). Tumor growth of VCaP xenografts was also significantly reduced by JJ-450, and JJ-450 was more potent than enzalutamide (Figure 7D). These results suggest that JJ-450 could inhibit CRPC xenografts expressing AR variants *in vivo*.

Discussion

The present study provides evidence that JJ-450 represents a novel class of AR antagonists capable of inhibiting both full-length AR and AR splice variants lacking LBD. These results show this class of small molecules can directly bind to AR, block AR recruitment to AREs,

and inhibit AR-target gene expression, including those induced by AR splice variants. Furthermore, JJ-450 could inhibit the growth of several AR-positive xenograft prostate tumor models, including enzalutamide-resistant 22Rv1 tumors.

In the RNA-seq study, (–)-JJ-450 inhibition of AR-target genes was almost identical to enzalutamide, which is a superb AR antagonist with high specificity (8). This finding together with the pulldown of purified AR by a JJ-450 and IMTPPE analog, **403**, strongly argues that JJ-450 targets AR directly and specifically. Thus, the JJ-450 chemotype could be used to develop a new class of highly specific AR antagonists.

Although both enzalutamide and JJ-450 exhibited similar specificity in AR inhibition based on the RNA-seq analysis in the LNCaP model, these two small molecules bind to different sites on AR. Enzalutamide and all the other FDA-approved AR antagonists target the LBD of AR and they do not affect AR splice variants or AR mutants lacking LBD. In contrast, JJ-450 and its analogs could inhibit full-length AR, ARv7 and NAR, a mutant AR that lacks LBD, suggesting that JJ-450 inhibition of AR was mediated through the NTD and/or DBD. IMTPPE had no effect on ³H-DHT binding to transfected GFP-AR in AR-negative PC3 cells (see Figure 1E), suggesting that IMTPPE does not influence DHT for binding to the ligand-binding domain (LBD) of GFP-AR in PC3 cells. However, IMTPPE could bind to other domain(s) of AR such as N-terminal domain (NTD) and/or DNA-binding domain (DBD) to inhibit PSA-luciferase induction by GFP-ARv7 in PC3:GFP-ARv7 (see Figure 3D), which contains NTD and DBD, but lacks LBD. However, we cannot rule out the possibility that JJ-450 may also interact with LBD, since it inhibited DHT binding to AR in C4-2 cells. However, JJ-450 did not inhibit DHT binding to transfected GFP-AR in PC3 cells. One possible explanation is that AR is hypersensitized to DHT in C4-2 cells (54) but not in PC3 cells, and JJ-450 may inhibit AR hypersensitization via targeting the NTD of AR. Another possibility is that the AR in C4-2 is mutated (55) whereas transfected GFP-AR in PC3 is wild-type. Future experiments will be required to conclusively determine whether IMTPPE and JJ-450 directly interact with AR, and further studies will be needed to address why JJ-450 inhibited DHT binding to AR in C4-2 cells but not to GFP-AR in PC3 cells.

The studies using the LNCaP xenograft tumor model show that JJ-450 can suppress AR function *in vivo*. JJ-450 suppresses serum PSA normalized to tumor volume in mice bearing LNCaP tumors, indicating that JJ-450 suppresses PSA synthesis on a per cell basis in LNCaP tumors. This is further supported by JJ-450 suppression of PSA mRNA expression in LNCaP tumors. Taken together, these findings suggest that JJ-450 inhibition of LNCaP tumor growth is mediated through AR inhibition.

In this study, JJ-450 inhibited AR-positive xenograft prostate tumors, including 22Rv1 xenograft tumors which express ARv7 and are resistant to LBD targeting AR antagonists including enzalutamide (45). In our experiments, JJ-450 appeared to inhibit LNCaP xenografts more effectively than 22Rv1 xenografts (see Figure 6F & Figure 7A). This difference may reflect that AR in LNCaP can be more effectively inhibited by JJ-450 than the AR in 22Rv1. 22Rv1 cells express both full-length AR and AR splice variants including ARv7 that lacks LBD. Figure 3C showed that JJ-450 at high dose (30 μM), but not low dose (10 μM) inhibited ARv7 activity. Thus, JJ-450 inhibition of ARvs may not be as potent as its

inhibition of full-length AR. One major reason for 22Rv1 being less sensitive to JJ-450 than LNCaP is likely that 22Rv1, but not LNCaP, cells express ARvs (56,57). According to the literature, enzalutamide can inhibit LNCaP but not 22Rv1 xenograft (58,59). It is thought that ARvs in 22Rv1 are responsible for the resistance to enzalutamide (45). JJ-450 and enzalutamide may target different domains of AR and the mechanisms leading to enzalutamide resistance may not confer JJ-450 resistance. Accordingly, it might be feasible to further develop JJ-450 to treat mCRPC resistant to enzalutamide. Since JJ-450 and enzalutamide target different regions in AR, the combination of JJ-450 with enzalutamide or any LBD-targeting AR antagonist could be synergistic, and it may be more difficult for AR to develop simultaneous resistance to both JJ-450 and enzalutamide.

In summary, this work suggests that JJ-450 is a promising clinical lead compound for the future development of a new class of AR antagonists that can target AR and AR splice variants lacking LBD. Further studies should focus on the determination whether JJ-450 analogs and AR directly interact, development of JJ-450 analogs that have sufficient oral bioavailability and favorable pharmacokinetics, as well as mechanistic studies defining the exact binding site(s) of JJ-450 on AR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We are grateful to Megan Lambert, Robin Frederick and Aiyuan Zhang for technical support.

Financial Support:

This work was funded in part by DOD Award W81XWH-16-1-0659, NIH Grants R01 CA186780 (Z.W.), P50 CA180995 (Z.W.), and R50 CA211242 (L.E.P.) as well as by UPMC Enterprises. This project used the UPMC Hillman Cancer Center Animal Facility, the Tissue and Research Pathology Services/Pitt Biospecimen Core, and the Genomics Research Core (GRC) and Cancer Bioinformatics Services which were supported in part by NCI award P30 CA047904 and the Senior Vice Chancellor's Office at University of Pittsburgh.

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Figure 1.

IMTPPE scaffold directly binds to AR. (A) Structures of IMTPPE, **26b**, **325**, **325**-conjugated beads (**403**) and control beads (**403c** and **974**). (B) GFP-AR, but not GFP-Rb1, was pulled down by **403**. C4-2 cells were transiently transfected with GFP-AR or GFP-Rb1 vectors for 48 h in complete RPMI medium before collecting whole cell lysates. Cell lysates were incubated with 50-fold IMTPPE or DMSO for 2 h at room temperature prior to incubation with beads (**403**, **403c** or **974**) at 4 °C for 4 h. The pulldown (left panel) and lysate input (right panel) were analyzed by immunoblot using anti-GFP antibody. (C) C4-2 cells were cultured in complete RPMI medium with 1 nM R1881 for nuclear extract preparation. Equal amount of nuclear extracts was incubated with agarose beads **403** or **403c**

in the presence or absence of 50-fold IMTPPE at 4 °C overnight. The pulldown samples were analyzed by immunoblot using anti-AR antibody. (D) Purified AR protein was pulled down by **403**. Equal amount of human AR protein from Sigma was incubated with agarose beads **403** or **403c** at 4 °C overnight. The pulldown samples were analyzed by immunoblot using anti-AR antibody. (E) Effect of IMTPPE on DHT binding to AR in radioactive ligand binding assay. ³H-DHT binding to AR in cytoplasmic extracts of C4-2 and PC3 cells stably transfected AR expression vector (PC3-AR) were determined in the presence DMSO or 10 μM IMTPPE at 4 °C for overnight. Percentage of ³H-DHT bound to AR in lysates was calculated. Cytoplasmic extracts of PC3 cells was served as control. Results represent mean ± SD and are representative of at least 2 biological replicates. *p<0.05; **p<0.01.

Figure 2.

JJ-450 inhibited AR recruitment to its target genes and AR transcriptional activity. (A) Chemical structures of IMTPPE, (+)-JJ-450, and (-)-JJ-450. (B) C4-2-PSA-rl cells (24), a C4-2 subline containing a PSA-luciferase reporter vector (pPSA6.1-Luc) and a Renilla luciferase reporter vector (pRL-TK), cultured in complete RPMI medium, were treated with 1 nM R1881 in the presence of vehicle or indicated concentrations of (+)-JJ-450 or (-)-JJ-450 for 24 h prior to luciferase assays. Firefly luciferase values were determined and normalized to Renilla. (C) C4-2-PSA-rl cells were treated with indicated concentrations of R1881 in the presence of DMSO, 10 μM (+)-JJ-450, (-)-JJ-450, or enzalutamide for 24 h prior to luciferase assays. (D) Western blot analysis of (-)-JJ-450 and (+)-JJ-450 inhibition

of PSA expression in LNCaP, C4-2, LAPC4 and 22Rv1 cells. Cultured cells were treated with DMSO, (+)-JJ-450, or (–)-JJ-450 in complete RPMI/IMDM medium at indicated concentrations for 24 h. GAPDH was probed as sample loading control. (E) ChIP analysis of JJ-450 inhibition of R1881-induced AR binding to AREs in C4-2 cells. C4-2 cells were cultured with cFBS, phenol red-free RPMI medium and treated with 1 nM R1881 and DMSO, 10 μ M IMTPPE, or JJ-450 for 2 h before ChIP assays. JJ-450 and IMTPPE inhibited AR recruitment to the AREs in the PSA enhancer region (PSA-EN-ARE) and in TMPRSS2 promoter region, but not in the PSA promoter region (PSA-PR-ARE). Results represent mean $\pm$ SD and are representative of at least 3 biological replicates. * p <0.05; ** p <0.01, *** p <0.001, **** p <0.0001.

Figure 3.

JJ-450 inhibits LBD-independent transcriptional activity of AR. ARv7-positive 22Rv1 cells were transiently co-transfected with pPSA6.1-Luc and pRL-TK reporter vectors in cFBS, phenol red-free RPMI medium for 24 h and then treated with indicated concentrations of (+)-JJ-450, (-)-JJ-450 or enzalutamide in the presence (A) or absence (B) of R1881 for additional 24 h prior to luciferase assays. (C) C4-2 cells were transiently transfected with pPSA6.1-Luc and pRL-TK reporter vectors, together with GFP, GFP-ARv7 or GFP-NAR (mutant AR lacking LBD) expression vectors for 24 h in cFBS, phenol red-free RPMI medium, then treated with indicated concentrations of (+)-JJ-450, (-)-JJ-450 or enzalutamide for additional 24 h under androgen-depleted condition before luciferase

assays. Parallel western blot data was showed in Supplementary Figure S5A. (D) GFP-ARv7:PC3, a PC3 subline stably transfected GFP-ARv7 expression vector (28), was transiently co-transfected with pPSA6.1-Luc and pRL-TK reporter vectors for 14 h in cFBS, phenol red-free RPMI medium, then treated with indicated concentrations of (+)-JJ-450, (-)-JJ-450 or enzalutamide for additional 24 h before luciferase assays. 22Rv1 cells were cultured in cFBS, phenol red-free RPMI medium for 24 h, then treated with indicated concentrations of (+)-JJ-450, (-)-JJ-450 or enzalutamide for additional 24 h prior to qRT-PCR (E) or immunoblotting analysis (F). UBE2C protein expression was quantified relative to GAPDH for each lane using Image lab (Bio-Rad, Hercules, CA, USA). The same experiment was also performed using another cell line CWR-R1 (Supplementary Figure S5B). Results represent mean $\pm$ SD and are representative of at least 3 biological replicates. * $p < 0.05$; ** $p < 0.01$.

Figure 4.

(-)-JJ-450 and (+)-JJ-450 do not inhibit estrogen receptor α (ER α) or glucocorticoid receptor (GR) in C4-2 cells. C4-2 cells were transiently co-transfected with Renilla luciferase reporter and 3x-ERE-TATA luciferase reporter (46), together with GFP or GFP-ER α expression vectors for 24 h in cFBS, phenol red-free RPMI medium, then treated with 1 nM 17 β -estradiol (E2) and indicated concentrations of (+)-JJ-450 or (-)-JJ-450 for additional 24 h for luciferase assays (A) or western blot (B). Similarly, C4-2 cells were transiently co-transfected with Renilla luciferase reporter and MMTV-firefly luciferase reporter (36), together with GFP or GFP-GR expression vectors for 24 h in cFBS, phenol red-free RPMI medium, then treated with 50 nM dexamethasone (Dex) and indicated concentrations of (+)-JJ-450/(-)-JJ-450 for additional 24 h for luciferase assay (C) or western blot (D). Results represent mean $\pm$ SD and are representative of at least 3 biological replicates.

Figure 5.

(-)-JJ-450 inhibits AR-dependent transcriptome specifically. RNA-seq analysis was performed to determine the effect IMTPPE, (-)-JJ-450, (+)-JJ-450, and enzalutamide on R1881-induced transcriptome in cultured LNCaP cells. Differentially expressed genes were defined as those showing absolute fold change ≥ 2 and FDR ≤ 0.05 . R: R1881; (+)450: (+)-JJ-450; (-)450: (-)-JJ-450; Enz: Enzalutamide. (A) Multidimensional scaling analysis of the whole gene expression program. (B) Heat-map of the whole gene expression program. Heatmap of selected 25 androgen responsive genes was provided in Supplementary Figure S7. (C) Venn diagram analysis of differential expression genes among three groups: enzalutamide vs IMTPPE, enzalutamide vs (+)-JJ-450 and enzalutamide vs (-)-JJ-450. More

details are provided in Table S2. (D) Venn diagram analysis of genes expression between two groups: Control vs enzalutamide and Control vs (–)-JJ-450. More details are provided in Supplementary Table S3. Experiments were performed on 3 biological replicates for each group.

Figure 6.

JJ-450 inhibited growth and expression of AR-target genes in relapsed LNCaP xenograft tumors in castrated mice. JJ-450 or enzalutamide inhibited the short-term growth of relapsed LNCaP tumors (A) and serum PSA normalized to tumor volume in mice (B) at day 3 and 6 after the treatment. (C) Representative immunohistochemistry images of LNCaP xenografts in (A) and stained for Ki67, cleaved caspase-3 (Cas-3), AR and hematoxylin and eosin (H&E). Size bars in the image indicate 100 μ m. (D) Ki-67 positive cell density was determined for LNCaP xenograft tumors. JJ-450 and enzalutamide significantly reduced proliferation compared with control. Student's t-test. Mean $\pm$ SD. (E) Relative PSA and FKBP5 mRNA level in xenografts at day 6. (F) JJ-450 inhibited long-term growth of

relapsed LNCaP xenograft tumors in castrated mice. Castration was performed when LNCaP xenograft tumor diameter reached to 7-8 mm. Mice were randomized and treated with JJ-450 (o.g 10 mg/kg body weight, i.p. 10 mg/kg body weight, o.g. 75 mg/kg body weight) six days on, one day off, started 10-22 days after castration. Mean $\pm$ SD. (G) Kaplan–Meier curves of mice described in (F) showing the effect of JJ-450 on the survival of LNCaP tumor bearing mice. Significance was determined using a Log-rank Mantel-Cox Test. Results represent mean $\pm$ SD, number of animals indicated in parentheses. * p <0.05, ** p <0.01, *** p <0.001. Enz: Enzalutamide.

Figure 7.

JJ-450 inhibited growth of relapsed xenograft tumors that express AR splice variants in castrated mice. (A) Mice with 22Rv1 xenograft were castrated when tumor diameter reached to 5 mm. Mice were randomized and treated with JJ-450 (i.p. 10 mg/kg body weight, i.p. 75 mg/kg body weight) every day at the time of castration. (B) Body weight of animals described in (A) over the duration of the experiment. (C) Kaplan–Meier curves of mice described in (A) showing the effect of JJ-450 on the survival of 22Rv1 tumor bearing mice. Significance was determined using a Log-rank Mantel-Cox Test. (D) Mice with VCaP xenograft were castrated when tumor volume reached to ~250 mm³. Mice were randomized and treated with JJ-450 (i.p. 75 mg/kg body weight) or enzalutamide (i.p. 10 mg/kg body weight) every day, started two weeks after castration. Results represent mean $\pm$ SD, number of animals indicated in parentheses.