Title: Discovery of JNJ-63576253, a next-generation androgen receptor antagonist active against wild-type and clinically-relevant ligand binding domain mutations in metastatic castration-resistant prostate cancer (mCRPC)

Running Title: Discovery of JNJ-63576253, a next-generation AR antagonist

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Conflicts of Interest: All authors are current employees, former employees, or contractors of Janssen Research and Development.

Abstract

Numerous mechanisms of resistance arise in response to treatment with second-generation androgen receptor pathway inhibitors in metastatic castration-resistant prostate cancer (mCRPC). Amongst these, point mutations in the ligand binding domain can transform antagonists into agonists, driving the disease through activation of androgen receptor (AR) signaling. In order to address this unmet need, we report the discovery of JNJ-63576253, a next-generation androgen receptor pathway inhibitor that potently abrogates AR signaling in models of human prostate adenocarcinoma. JNJ-63576253 is advancing as a clinical candidate with potential effectiveness in the subset of patients who do not respond to or are progressing while on 2nd-generation AR targeted therapeutics.
Introduction

An estimated 31,620 men died from Prostate Cancer in 2019, representing 10% of all cancer-related deaths [1]. Nevertheless, over the past 20 years mortality has decreased nearly 50% [1], partly attributable to the development of androgen receptor (AR) pathway inhibitor therapy, abiraterone acetate plus prednisone (AAP) [2], and second-generation AR antagonists enzalutamide (MDV-3100) [3] and apalutamide (ARN-509) [4]. Patient progression while on these therapies has been associated with numerous resistance mechanisms. These include mutations specific to the AR signaling pathway (AR aberrations): gene [5, 6] or enhancer [7] amplification, genomic structural rearrangement [8], splice variant isoform expression [9], ligand binding domain (LBD) point mutations [10, 11], adrenal and intraprostatic androgen synthesis [12-14], and glucocorticoid receptor bypass [15, 16]. Treatment-emergent small-cell neuroendocrine (t-SCNC) [17] and double-negative (DNPC) [18] prostate cancer, in which prostate lineage specificity is lost in response to AR pathway inhibition, have arisen more recently as mechanisms through which drug resistance can occur.

Mutations that occur in the ligand binding domain in response to AR pathway inhibitors are one mechanism through which therapeutic resistance arises. While absent in non-metastatic castration-sensitive patients [19], LBD mutations are enriched in heavily-treated mCRPC [5, 20], suggesting that they are beneficial to the propagation of clonal populations harboring them. In cellular models, the T878A mutation, endogenous to LNCaP, activates AR signaling and proliferation in response to treatment with flutamide [21]. Similarly, bicalutamide, dehydroepiandrosterone (DHEA), and corticosteroids act as agonists in the presence of W742C/L [22], H875Y [23], and L702H [24] mutations respectively.

In 2013, the AR F877L mutation was reported to cause an antagonist-to-agonist switch in response to treatment with both enzalutamide [11] and apalutamide [10] that increased transcriptional activation of the androgen receptor and lead to drug resistance. In the clinic, AR F877L was detected in the plasma ctDNA of 3/29 progressing patients enrolled in the ARN-509 Phase I trial. Combined with expansion cohort data, 14% of patients possessed a point mutation in the ligand binding domain at progression [25], demonstrating an unmet need.

In this report, we characterize JNJ-63576253, as a potent and selective next-generation AR pathway inhibitor of AR wild-type, AR F877L, and other clinically detected ligand binding domain mutations. We demonstrate that this molecule inhibits transcriptional activity in reporter assays, cellular proliferation, and androgen receptor downstream target gene expression. Furthermore, we show that in an enzalutamide-resistant LNCaP F877L xenograft model, JNJ-63576253 causes tumor growth inhibition. JNJ-63576253 is currently being evaluated in a Phase 2 clinical trial (NCT02987829).
Materials and Methods

Cell Culture and Reagents

HepG2 (ATCC Cat# HB-8065, RRID:CVCL_V331), VCaP (ATCC Cat# CRL-2876, RRID:CVCL_2235), and PC3 (ATCC Cat# CRL-7934, RRID:CVCL_0035) were obtained from ATCC. No further authentication was performed. LNCaP AR/cs and LNCaP F877L, originally derived from LNCaP.FGC (ATCC Cat# CRL-1740, RRID:CVCL_1379), were obtained from Aragon Pharmaceuticals. The authenticity of the LNCaP lines was verified by short tandem repeat (STR) analysis at Analytic Biological Services (ABS, Delaware). All cell cultures were verified mycoplasma-free weekly using the Lonza MycoAlert Kit (LT07-418). Cellular assays utilized cultures that had been passaged 10 or fewer times. The chemical synthesis of JNJ-63576253 is discussed in Zhang et al. (submitted). Enzalutamide was synthesized according to published procedures and stored at a stock concentration of 10nM in DMSO. R1881 was obtained from Sigma and stored at a stock concentration of 10mM in DMSO. Stock compounds were kept in a dessicator and replaced every two weeks. Plasmids were obtained from Aragon Pharmaceuticals (Supplemental Figure 5).

Stable Reporter Cell Line Generation

LNCaP F877L and LNCaP AR/cs were transfected with a consensus sequence androgen response element firefly luciferase reporter (Qiagen) and selected under puromycin (Thermo) to generate a stable cell lines, referred to as LNCaP F877L ARE Luc and LNCaP AR/cs ARE Luc.

Transcriptional Reporter Assays

HepG2 cells were co-transfected with an androgen receptor response element firefly luciferase reporter and AR-VP16 construct for 24 hours. For AR-VP16 assays, 25,000 cells from the pooled transfection were seeded into compound and 90pM R1881-spotted white and clear bottom 96-well plates and incubated for 48 hours. AR F877L-VP16 assays utilized 1nM R1881. LNCaP reporter lines were seeded at a density of 10,000 cells per well, incubated overnight, then treated for 24 hours with compound in the presence of 0.1nM R1881. After treatment, cells were assayed using the SteadyGlo-luciferase kit (Promega) and read on an EnVision plate reader in luminescence mode. All reporter assays were conducted in media containing charcoal-stripped FBS. Raw data was analyzed in GraphPad Prism using the variable slope four parameter non-linear regression and normalized to vehicle (0% Activity) and R1881 (100% Activity) treatment.

Proliferation Assays

LNCaP AR/cs, LNCaP F877L, VCaP, and PC3 were seeded at densities of 5000 or 250 cells per well into 96-well plates and incubated overnight. Following compound treatment, the cells were incubated for six days before assessing proliferation using the CellTiter-Glo kit (Promega) and read on an EnVision plate reader in luminescence mode. All proliferation assays were conducted in media containing charcoal-stripped FBS. Raw data was analyzed in GraphPad Prism using the variable slope four parameter non-linear regression and normalized to compound treatment day samples (0% Activity) and R1881 (100% Activity) treatment.

Real-Time PCR

1x10^6 LNCaP AR/cs or LNCaP F877L were seeded into each well of a 6-well plate in RPMI-1640 supplemented with 10% charcoal-stripped serum for 24 hours. The cells were treated with 0.1nM R1881...
plus compound in dose-response for 24 hours following treatment. The cells were harvested and processed using a Qiagen RNeasy-Plus kit utilizing the QiaCube system. cDNA was synthesized using the High Capacity cDNA Reverse Transcriptase Kit (Thermo) and run on a Viia7 RT-PCR instrument (Thermo) using Thermo Taqman primers (KLK3 primer: Hs02576345_m1; FKBP5 primer: Hs01561006_m1). Statistical analyses, where applicable, were performed using ANOVA in GraphPad Prism.

**High-Content Imaging Cellular Localization**

Cells were seeded at 10,000 cells/well in PDL-coated 96-well plates (Greiner 655946) in androgen deprived medium: RPMI 1640 without phenol red (Gibco 32404-014), 5% charcoal stripped serum (Sigma F6765), and 2 mM L-Glutamine (Sigma G7513) for 24 hours. Dose-response testing was done at 0.0114, 0.0343, 0.103, 0.308, 0.925, 2.78, 8.35, and 25 μM with constant 0.5% DMSO and in the presence and absence of 1 nM methyltrienolone (R1881, ACC API0003347) added 1 hour after compound addition.

After 24 hours incubation at 37°C, cells were fixed with formaldehyde (5% final at RT) and permeabilized with ice-cold methanol. Hoechst 33258 (Invitrogen H1399) was added at final 2 μg/ml and CellMask Deep Red at 0.5 μg/ml (Invitrogen H32721). AR was detected by indirect immunofluorescence with a mouse anti-AR IgG (Abcam 49450), and Alexa Fluor 488 labelled goat anti-mouse IgG secondary (Invitrogen A-10001). PSA was detected using a rabbit anti-PSA IgG (Cell Signaling Technologies 5365S) and an Alexa Fluor 568 goat anti-rabbit IgG secondary (Invitrogen A-11011). Plates were imaged with CV7000 (Yokogawa), using a 20x objective. Excitation lasers and emissions filters for the fluorescent labels mentioned above were 405 nm and 445/45, 635 nm and 676/79, 488 nm and 525/50, and 561 nm and 600/37, respectively, and corresponding exposure times 120 ms, 120 ms, 500 ms and 500 ms. Data was collected from approximately 1200 cells per well for the DMSO controls. The experiment was performed twice with 3 technical replicates for a total of 6 replicates per compound dose-response.

Image analysis was performed with a custom script written in PerkinElmer Acapella. The Hoechst channel was used to segment the nuclei, and the CellMask Deep Red channel to define the cell area. “AR levels” and “PSA levels” were defined as the mean intensities in the whole cell region in the AR and PSA channel, respectively. AR translocation was defined as the ratio nuclear/whole cell total intensity in the AR channel. The three measurements were each aggregated per well by taking the median over the cells in the well. Visual QC and data normalization were performed in Phaedra [26].

**Animals**

All experimental procedures were conducted in accordance with Janssen’s Institutional Animal Care and Use Committee and U.S. Department of Agriculture regulations. For the Hershberger assay, pre-pubertal, 42-45 days of age, castrated male rats (Sprague Dawley, RRID:RGD_737903) were housed in standard caging. For LNCaP xenograft studies, six- to 8-week-old castrated male SCID Hairless Outbred (SHO*) were housed in sterile-ventilated caging. All animals were maintained under aseptic and under pathogen-free conditions. The animal holding room provided 12 hours of alternating light and dark cycles and met the standards of the Association for Assessment and Accreditation of Laboratory Animal Care specifications. Reverse osmosis water and autoclaved food were supplied *ad libitum*. Drugs were administered by individual body weight for each rat or by per fixed dose for each mouse. JNJ-63576253 was formulated in 20% Hydroxypropyl beta cyclodextrin (HPBCD); enzalutamide in 1% carboxymethyl cellulose (CMC), 0.1% Tween80, 5% DMSO; Testosterone propionate (TP) in corn oil.
Hershberger assay

Rats were administered JNJ-63576253 orally (po), once daily (qd) at 10, 30, or 50 mg/kg twice daily (bid) at a volume of 5 ml/kg together with testosterone propionate (TP) at 0.4 mg/kg qd at a volume of 0.5 ml/kg, sc for 10 days (n = 6/group). On study day 0 (pre-dose), day 4 or 5 (1-2 hours post dose) and day 11 (24 hours post last dose), blood was collected as part of the necropsy for three animals per cohort (n=6/group). At necropsy (day 11), the following androgen sensitive male accessory organs (ASO) were removed and weighed: Cowper’s gland (CP), seminal vesicles and coagulating glands (SVCG), glans penis (GP), ventral prostate (VP), and levator ani-bulbocavernosus (LABC).

Tumor xenograft efficacy studies

Mice were injected subcutaneously with LNCaP AR F877L mutant or wild-type AR human prostate tumor spheroids. The spheroids were generated by adding 200,000 cells to 0.5ml of Cultrex BME (Trevigen) and incubating for one week at 37°C with the surface complete media (RPMI-1640 plus 10% HI FBS) changed daily. Approximately 0.5ml was injected subcutaneously into the right flank of each mouse. When tumors were established (~250 mm³), mice were randomized into experimental groups (n=10/group) and treated orally with JNJ-63576253 at 30 or 50 mg/kg or enzalutamide at 30 mg/kg po, qd for 3-4 weeks. The tumor take rate was 50-70%. Terminal blood and tumor samples were collected at 2, 4, 7 or 24 hours post last dose of vehicle or JNJ-63576253 treatment. Tumor volumes and body weights were recorded twice per week using a digital caliper and analytic scale, respectively. Tumor volumes were calculated using the formula: Tumor Volume (mm³) = (a x b²/2); where ‘a’ represents the length, and ‘b’ the width.

Statistical analysis

For the Hershberger assay, the effects of JNJ-63576253 on change in weight of 5 different androgen sensitive organs (ASO): Cowper’s gland (CG), seminal vesicles and coagulating gland (SVCG), glans penis (GP), ventral prostate (VP) and levator ani-bulbocavernosus (LABC). Statistically significant suppression of ASO is required in 2 of 5 organs for a compound to be classified as an anti-androgen. Analysis was performed by t-test/ Mann-Whitney. Performance criteria is determined by calculating the Maximum Coefficient of Variation (%CV) for each of 5 ASO tissues. Maximum %CV has been established for androgenic/ antiandrogenic effects on dependent tissues for the castrate model based Endocrine Disrupter Screening Program validation studies [27]. If %CVs are not homogenous, then non-parametric statistical procedures are utilized. For the efficacy studies, tumor volume and body weight data were graphically represented and statistically analyzed utilizing GraphPad Prism software (RRID:SCR_002798). Statistical significance was evaluated for androgen receptor antagonist small molecule treated groups vs. vehicle-treated controls on the last day of the study using a 1-way or 2-way ANOVA with Dunnett’s multiple comparisons post-test or using a T-test for single comparisons. Differences between groups were considered significant when the probability value (p) was ≤ 0.05.

Molecular Modeling

A homology model of AR LBD was built using GR as a template and induced-fit docking calculations were performed using Schrödinger Suite 2014, using default settings [28], followed by manual docking and rotamer selection to further fine tune a “Bicalutamide-like” pharmacophore of the A-ring having the CN and CF3 groups. The modeled binding mode of JNJ-63576253 suggests that this
molecule can stabilize a putative “antagonistic” open Helix 12 conformation (orange tubes), while an “agonistic” closed conformation of Helix 12 (cyan ribbons), as depicted by superimposed agonistic conformation of AR LBD would have severe clashes with such a binding mode [29]. With this model, we have hypothesized that a potential agonistic conformational switch introduced by the F877L mutation (or other, pharmacologically similar LBD mutations) would be incompatible with binding of JNJ-63576253, likely due to the presence of an extended and bulkier piperidine moiety in this molecule (compared to enzalutamide).
Results

**JNJ-63576253 is a Potent and Selective Antagonist of Human Androgen Receptor Signaling In Vitro**

JNJ-63576253 (Figure 1a) was identified following extensive optimization of existing Janssen chemical matter [30, 31] with the aim of restoring activity against enzalutamide- and apalutamide-resistant cellular models driven by the F877L and other clinically relevant ligand binding domain point mutations [10, 11]. In biochemical assays, JNJ-63576253 displayed superior activity to enzalutamide in wild-type androgen receptor competitive radioligand binding assays with the synthetic androgen R1881. Competition for binding to estrogen and glucocorticoid receptors was undetectable or had IC\(_{50}\) greater than 30\(\mu\)M (Table 1), representing an approximately 1000-fold selectivity versus other nuclear hormone receptors. Utilizing molecular docking models informed by published data for other nuclear receptors, we confirmed that mutation from phenylalanine to leucine at AR amino acid 877 reduces steric hindrance in the ligand binding pocket. Subsequent binding of antagonists, such as enzalutamide, produces an agonist conformation rather than antagonist conformation (Figure 1b). This mechanism is similar to that which has been described computationally for enzalutamide and the flutamide and bicalutamide associated mutations at T878A and W742C/L [32-34].

To evaluate AR functional activity in cells, we utilized a fusion construct of AR and the VP16 virion phosphoprotein transactivation domain as a tool to sensitively discriminate ligand-receptor agonism [10, 35]. VP16 drives constitutive nuclear translocation and transactivation in the absence of coactivator recruitment [36]. In HepG2 transcriptional reporter models, JNJ-63576253 completely inhibited transiently transfected VP16-AR F877L (IC\(_{50}\) = 15nM) in the presence of 90pM R1881. In contrast, enzalutamide failed to reach 50% inhibition at any tested concentration up to 30\(\mu\)M (Figure 2a). JNJ-63576253 and enzalutamide displayed similar inhibition of AR wild-type VP16 in the presence of 90pM R1881 (Figure 2c). In VP16-AR F877L cells, enzalutamide elicited activation of the androgen receptor in the absence of R1881 at concentrations as low as 1nM, reaching 40% activity at 3\(\mu\)M (Figure 2b). JNJ-63576253 did not significantly activate the androgen receptor in the absence of R1881, reaching 5% activity at 10\(\mu\)M with no activity at 30\(\mu\)M. These in vitro concentrations approximate tumor concentrations measurable at efficacious doses in pharmacodynamic and xenograft studies (Figure 5, 6).

We next sought to assess the activity of JNJ-63576253 in LNCaP, a model of prostate adenocarcinoma. In cells stably transfected with AR F877L and an androgen response element (ARE)-driven firefly luciferase reporter, JNJ-63576253 completely inhibited AR-mediated transactivation in the presence of 100pM R1881 (IC\(_{50}\) = 99nM). In contrast, enzalutamide acted as an incomplete antagonist, activating AR signaling at concentrations greater than 1\(\mu\)M (Figure 2d). Reporter activity reached 154% of control stimulation with 100pM R1881, the concentration that induces maximal proliferation in this model (Supplemental Figure 4). Both compounds were comparably effective at inhibiting AR transactivation in LNCaP AR/cs [37] stably expressing the ARE reporter at low nM IC\(_{50}\) concentrations (Figure 2e).

Additionally, JNJ-63576253 was capable of effectively inhibiting transactivation of other clinically-relevant AR ligand binding domain mutations in transiently transfected HepG2 (Figure 2f). We demonstrate that the F877L single mutation is sufficient to induce potent agonism in HepG2 transient expression models (Supplemental Figure 3).
**JNJ-63576253 Abrogates Cellular Proliferation, Nuclear Translocation, and AR Target Gene Expression in Models of Human Prostate Adenocarcinoma**

We investigated the effectiveness of JNJ-63576253 at inhibiting cellular proliferation in several models of human prostate adenocarcinoma. In LNCaP F877L overexpression models, JNJ-63576253 treatment in the presence of 100pM R1881 completely inhibited proliferation at concentrations greater than 3μM with an IC₅₀ of 197nM. As predicted by the reporter data, enzalutamide displayed incomplete antagonist activity at concentrations greater than 3μM (Figure 3a). Both compounds were nearly equivalent at inhibiting proliferation in the LNCaP AR/cs line with IC₅₀ values of approximately 250nM (Figure 3b). In the VCaP model of prostate adenocarcinoma, which contains an amplified AR, TMPRSS2-ERG fusion, and expresses AR-V7, enzalutamide and JNJ-63576253 displayed nanomolar inhibition of proliferation (IC₉₀ < 100nM, Figure 3c).

Next, we used RT-PCR to assess the ability of JNJ-63576253 and enzalutamide to inhibit the transcription of two canonical AR downstream target genes, KLK3 and FKBP5. Utilizing the LNCaP AR F877L overexpressing model, both compounds inhibited transcription in the presence of R1881. However, the inhibitory effect of enzalutamide was blunted with increasing concentrations such that KLK3 and FKBP5 expression at 30μM was 71% and 133%, respectively, of 100pM R1881-only treatment. These results contrast with a reduction in KLK3 and complete inhibition of FKBP5 gene expression by JNJ-63576253 (Figure 4a, 4b). We discovered that in the absence of ligand, enzalutamide at 30μM was capable of recapitulating 86% and 141% of 100pM R1881-induced KLK3 and FKBP5 expression, respectively. JNJ-63576253 alone produced 25% of 100pM R1881-induced KLK3 expression at 30μM and no agonism for FKBP5 (Figures 4c and 4d). These data demonstrate that in an F877L mutated AR, enzalutamide acts as a partial antagonist in the presence of R1881 and an agonist in its absence. In contrast, JNJ-63576253 maintained antagonist activity both in the presence and absence of R1881. Both enzalutamide and JNJ-63576253 inhibited 100pM R1881 mediated AR downstream target gene expression in the LNCaP AR/cs model. There was no statistically significant difference between enzalutamide or JNJ-63576253 at identical concentrations (Supplemental Figure 2).

Androgen receptor nuclear translocation occurs following ligand binding and intramolecular dimerization [38]. The first-generation anti-androgen bicalutamide was shown to reduce the ratio of nuclear-to-cytoplasmic AR in LNCaP by 50%, whereas, enzalutamide and apalutamide inhibited nuclear AR to approximately 10% of total [36]. To assess inhibition of nuclear translocation, we employed an immunofluorescent imaging assay to measure AR protein localization in LNCaP following compound treatment for 24 hours. JNJ-63576253 reduced nuclear localization in the presence of R1881 to 9% of total protein at 8.25μM while enzalutamide reduced it to 35% (Supplemental Figure 8a, 8c). PSA protein was reduced indicating inhibition of AR downstream gene transcription. Employing an enzalutamide-resistant LNCaP line containing a heterogeneous mixture of mutations, AR nuclear localization was reduced to 30% of total AR protein by JNJ-63576253 at 8.25μM in contrast to 64% by enzalutamide (Supplemental Figure 8b). Both compounds reduced AR protein at μM concentrations in the absence of significant cell death (Supplemental Figure 8d).
JNJ-63576253 Inhibits LNCaP F877L Xenograft Tumor Growth and Male Accessory Sex Gland Size In Vivo

To confirm in vivo androgen antagonism dependent activity of JNJ-63576253, castrated male rats were treated with testosterone propionate (TP) (0.4 mg/kg SC) and either vehicle or JNJ-63576253 at 10, 30, or 50 mg/kg PO in the Hershberger assay. Administration of JNJ-63576253 with TP (0.4 mg/kg) at either 30 or 50 mg/kg was well tolerated and resulted in statistically significant inhibition of weight gain in Androgen Sensitive Organs (ASO) (Figure 5a). Both Seminal Vesicles and Coagulating Glands (SVCG) and Ventral Prostates (VP) were significantly reduced (<49% or <48%, respectively) as compared to the TP treated group (p<0.05). At the lowest 10 mg/kg dose level of JNJ-63576253 failed to inhibit TP-induced ASO weight gain. Notably, at 50 mg/kg, TP-stimulated ASO development was completely inhibited to that of the vehicle-treated castration controls. Furthermore, the degree of ASO weight suppression correlated with plasma drug concentrations (Fig. 6b) and drug exposures appeared linear across the dose range (Fig 6c). These data support the minimally effective dose of 30 mg/kg and a maximally effective dose of 50 mg/kg for JNJ-63576253 in the Hershberger assay.

Next, we tested if inhibition of androgen antagonism dependent activity, as measured by the degree and duration of TP-induced ASO weight gain, correlated with suppression of tumor growth in vivo. Male SHO castrated mice bearing established human prostate LNCaP AR/cs tumors were orally administered vehicle alone or JNJ-63576253 at 30 mg/kg once daily for three weeks. As shown in Figure 6a, daily administration of JNJ-63576253 elicited 78% tumor growth inhibition (TGI) (p<0.05) as compared with the vehicle treated controls. The effect of JNJ-63576253 on tumor growth was further evaluated in a second human prostate cancer xenograft model. Mice bearing established human LNCaP F877L tumors were orally administered vehicle alone or JNJ-63576253 at 30 or 50 mg/kg once daily for three weeks (Figure 6b). Administration of JNJ-63576253 resulted in a statistically significant tumor growth inhibition of > 58% TGI (p<0.01) as compared with the vehicle treated control mice. In contrast, no efficacy was observed with enzalutamide treatment at 30 mg/kg (Fig. 7c). Daily dosing with JNJ-63576253 was well tolerated at all dose levels tested with no adverse effect on body weight (Supplemental Figure 7). Plasma and tumor concentrations of JNJ-63576253 were determined at 2, 4, 7 and 24 hours after dosing. The peak concentration was 1745 or 3875 ng/ml and trough levels of drug was 250 or 310 ng/ml (for 30 or 50 mg/kg, respectively) (Figure 6d, 7e). Drug concentration was higher in the tissue with tumor to plasma (T/P ratio) of ~21-25-fold for both dose levels.
Discussion

Despite therapeutic advances in the past decade, an estimated 31,620 men died from prostate cancer in the United States in 2019 [1], representing a significant unmet need. First-generation therapies (e.g. flutamide [39] and bicalutamide [40]) were thought to ablate AR transcriptional activity by either directly preventing androgen binding to the ligand binding pocket of the AR or by reducing circulating plasma levels of androgens (e.g. leuprorelin [41]). Studies that emphasized the continued importance of the AR in disease progression through gene amplification [42] and antagonist-to-agonist conversion by amplification [37] laid the scientific rationale for the development of AAP, enzalutamide, and apalutamide. In the SU2C-PCF mCRPC cohort, 85% of patients progressing on these therapies possessed AR aberrations when including structural variants and the recently discovered amplified upstream putative enhancer [5, 6, 8]. These data illustrate that metastatic tumors are enriched in AR mutations and provide a strong rationale for the continued development of more effective inhibitors.

In response to this unmet need, we developed JNJ-63576253, a next-generation AR antagonist that displayed robust inhibition in wild-type and LBD mutated, enzalutamide-resistant models of prostate cancer. JNJ-63576253 attenuated androgen-sensitive organ growth in the rat Hershberger model (Figure 5) which demonstrated oral bioavailability and tissue exposure. We showed that in an enzalutamide-resistant LNCaP F877L xenograft model, JNJ-63576253 caused tumor growth inhibition (Figure 6). In a 93-cell line panel, JNJ-63576253 and enzalutamide displayed similar anti-proliferative activity profiles (Supplemental Figure 6). In the KINOMEscan assay used to detect activity against a panel of 468 kinases, JNJ-63576253 was not identified as a hit against any target (Supplemental Figure 7) [43]. Based on these data, we predict that JNJ-63576253 will be tolerated in the clinic and possess a similar profile to other marketed androgen receptor antagonists.

JNJ-63576253 is effective in models that possess AR amplification (LNCaP AR/cs) and AR-V7 expression (VCaP). AR amplification is enriched in mCRPC compared to treatment-naïve patient populations [5, 6, 19] and AR-V7 has been associated with resistance to enzalutamide and apalutamide [9]. In the clinic, AR-V7 is often expressed in the context of AR-FL copy number amplification and patients within this subpopulation often respond to apalutamide, suggesting that the full-length isoform drives transcriptional activity [44]. We hypothesized that this mechanism may explain the activity of JNJ-63576253 in the VCaP model. In contrast, AR structural isoforms may arise due to AR genomic rearrangement or deletion [8] and have been linked to enzalutamide resistance in vitro [45]. JNJ-63576253 was not tested in models that express these structural variants such as the 22Rv1 or R1-D567 [46].

We attributed the ability of enzalutamide to inhibit LNCaP F877L overexpressing lines at lower than physiological concentrations (less than 1μM) (Figures 2d, 3a) to the interplay of endogenous and mutant AR protein within the cell. In the HepG2 AR F877L reporter model, in which no wild-type protein is present, inhibition of AR signaling by enzalutamide is mitigated at all concentrations in comparison to JNJ-63576253 (Figure 2a). Additionally, in the LNCaP F877L xenograft model, a clear difference in tumor growth inhibition is observed between JNJ-63576253 and enzalutamide (Figure 6c). These results highlight that in interpreting antagonist-agonist switch by the F877L mutation, an understanding of in vivo physiological concentrations must be considered when interpreting in vitro assay data (Figure 6c).
In contrast to a previous report, we discovered that the ability of enzalutamide to activate AR F877L was not dependent on the presence of the T878A mutation [47]. This discrepancy may be explained by the different cellular overexpression models used to transfect HepG2 cells and resulting variations in transcriptional machinery between prostate and non-prostate cell lines. Molecular dynamics suggests that binding of enzalutamide to both the F877L and F877L/T878A mutations induces an agonist conformation [34]. Variations in reporter assay IC50s may be explained by the different AR constructs and R1881 concentrations utilized. These deviations in assays and methods were employed in order to draw more relevant comparisons with the previously published preclinical characterization of apalutamide [36].

AR ligand binding domain mutations have previously been correlated with overall and progression free survival in the post-docetaxel [48] but not in the treatment-naive space [49]. Furthermore, their detection in mCRPC [5] and absence in localized disease [19] suggests that their emergence is a consequence of ADT treatment-acquired resistance. Prior studies have linked the occurrence of LBD mutations with therapeutic intervention. The T878A mutation, in particular, has been detected in patients progressing on flutamide [50] and AAP or ketoconazole [51]. AR T878A detection in ctDNA was associated with shorter overall survival compared to wild-type tumors in patients that relapsed while on AAP [52]. In cellular models possessing the T878A or W742C/L mutations, both flutamide [21] and bicalutamide [22], respectively, are capable of rescuing AR signaling activity in the absence of exogenous androgen.

The recently discovered F877L mutation has been detected in patients treated with the second-generation antiandrogens enzalutamide or apalutamide [10, 25, 53, 54]. Like the T878A mutation, F877L enables agonism of the androgen receptor by 2nd-generation anti-androgens in the absence of androgen and partial antagonism in the presence of androgen [10, 11] (Supplemental Figure 1). Joseph et al. were the first group to describe the F877L mutation in 3/27 patients progressing after multiple cycles of apalutamide in the clinic [10]. Data from the expansion cohort confirmed an increase in the mutational frequency of F877L during treatment [25]. Interestingly, in this study 2 of 5 patients who presented with de novo AR F877L prior to therapy responded to apalutamide with corresponding decreases in PSA and prolonged time to radiographic progression. These results imply that the F877L mutation is not a mechanism of primary resistance; however, these patients possessed a low (<0.1%) mutation fraction at baseline suggesting that a critical threshold may be required. At progression, the F877L mutational fraction for these two patients had increased more than ten-fold. Continuing this trend, results from the SPARTAN Phase 3 registration trial of patients with localized disease reported that AR LBD mutations were present in 1.9% of ctDNA tested at baseline but increased to 8.8% by end-of-treatment [44]. However, progression-free survival 2 (PFS2) and overall survival (OS) was not associated with apalutamide treatment in patients that acquired these mutations. Indeed, the mutation rate was similar in both apalutamide plus ADT and ADT alone arms (7/69 and 5/69 respectively), suggesting that ADT, not apalutamide, drives the selection of LBD point mutations. In contrast, results from the TITAN Phase 3 trial evaluating the metastatic CRPC population reported that acquisition of LBD mutations was increased by end-of-treatment in 13.3% (2/15) of patients on apalutamide plus ADT in contrast to 6.82% on ADT alone (3/44) ([55]). In this cohort, the detection of AR aberrations, in general, was associated with both decreased PFS2 and OS. We speculate that in the localized setting apalutamide effectively abrogates AR signaling and suppresses bulk tumor growth. However, clonal populations possessing LBD mutations and/or other mutations eventually expand until they sufficiently dominate the tumor.
microenvironment to induce clinical progression. The fitness of metastatic tumors, possessing a greater mutational burden, is enhanced through further AR aberrations, including LBD mutations, producing a significant effect on PFS2 and OS outcomes.

The SU2C-PCF consortium reported that 21 of 150 (14%) patients in their mCRPC cohort possessed LBD point mutations, however, the F877L mutation was not detected [5]. Since 50% of the patients in this study had not received AAP or enzalutamide, this may have limited the F877L mutational frequency. Seven patients possessed the T878A mutation which has been associated with flutamide and AAP intervention and seven with the L702H mutation, which is activated by corticosteroids such as the prednisone component of the abiraterone treatment regimen. Prior flutamide and bicalutamide use was not disclosed in the study. Other groups have similarly reported ligand binding domain mutations in patients treated with enzalutamide and/or abiraterone acetate plus prednisone and their association with worse clinical outcomes [52, 53].

CRPC tumors remain dependent on the AR and both AR amplification and ligand binding domain mutations have been associated with worse clinical outcomes [48]. Efforts to delineate the role that each individually plays in tumor progression have been difficult. Several studies have suggested that AR amplification and ligand binding domain mutations do not occur in the same tumor [5, 54], while AR structural variants appear to be exclusive of LBD mutations but can be found in tumors harboring amplified AR [8]. Other studies have described interpatient heterogeneity with point mutations and gene amplification arising prior to and after AR gene amplification [56, 57]. These cohorts represent heterogeneous patient populations and larger studies will be required to ascertain in which context these are relevant. JNJ-63576253 is anticipated to be effective in tumor populations possessing amplified AR and LBD mutations. The role of AR variants in clinical outcomes remains unresolved [58-60]. As discussed previously, we predict that JNJ-63576253 could be effective in this setting depending on the context [45].

As the prostate cancer patient population continues to evolve in the next decade, existing data suggests that the AR LBD acquired mutation frequency is likely to increase with treatment [44, 55]. Although earlier intervention delays progression, ultimately AR aberrations will emerge that correlate with a worse prognosis [61, 62]. Addressing this growing unmet need, the data presented here support JNJ-63576253 as a clinical candidate with potential effectiveness against multiple AR pathway mechanisms of resistance in the subset of patients who do not respond to or are progressing on 2nd-generation AR targeted therapeutics. This agent is currently under investigation in a Phase 1/2A study with patients diagnosed with metastatic castration-resistant prostate cancer [NCT02987829, https://clinicaltrials.gov].
Acknowledgements

The authors would like to thank Brent Rupnow, Shibu Thomas, and Margaret Yu for scientific discussion related to the manuscript.
References


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Figure 1: (a) Chemical structure of JNJ-63576253. (b) Radioligand binding inhibition and affinity calculations were determined using [3H]-methyltrienolone, [3H]-dexamethasone and [3H]-estradiol for AR, GR, and ER, respectively. For ER, it was not possible to determine inhibition or affinity and data are scored as not calculated (NC). (c) A manual docking model of JNJ-63576253 (orange balls and sticks; gray surface) in complex with a potentially antagonistic conformation (orange tubes; homology model of AR built using GR PDB ID:1NHZ) of AR LBD. Superposition of a crystal structure of AR in agonistic conformation (cyan ribbons; PDB ID: 1T5Z) is shown as a reference to demonstrate the potential role of Helix 12 in modulating the pharmacology of AR.

Figure 2: JNJ-63576253 inhibits AR-mediated transactivation in reporter system model. AR F877L-VP16 were co-transfected with an ARE firefly luciferase reporter into HepG2 cells and treated with compound in the presence (a) or absence (b) of 90pM R1881, respectively, for 48 hours. (c) AR -VP16 plasmids were co-transfected with an ARE firefly luciferase reporter into HepG2 cells and treated with compound in the presence of 90pM R1881 for 48 hours. (d) LNCaP F877L and (e) LNCaP AR/cs ARE reporter stably transfected cell lines were seeded, incubated overnight, then treated for 24 hours with compound in the presence of 100pM R1881 in charcoal-stripped FBS. (f) HepG2 were co-transfected with either the W875Y, T878A, or L702H mutant AR and an ARE firefly luciferase reporter and treated for 48 hours with compound. The data was collated from at least 3 biological replicates obtained from at least 3 independent experiments. Data are expressed in mean ± SEM.

Figure 3: JNJ-63576253 inhibits cellular proliferation AR-driven cell lines. (a) LNCaP F877L, (b) LNCaP AR/cs, and (c) VCaP were incubated for 6 days in the presence of compound and R1881 in charcoal-stripped FBS. The data was collated from at least 3 biological replicates obtained from at least 3 independent experiments. Data are expressed in mean ± SEM.

Figure 4: JNJ-63576253 inhibits expression of AR downstream target genes KLK3 (a) and FKBP5 (b) in LNCaP F877L in the presence of R1881. In the absence of R1881, JNJ-63576253 does not activate gene expression (c,d). The data was collated from 4 biological replicates obtained from 2 independent experiments. Data are expressed in mean ± SEM.

Figure 5: For the Hershberger study, peripubertal male Sprague Dawley (SD) rats were castrated at 42-45 days of age and weighed on average 200-220 g were randomized into experimental groups by body weight 11 days post-castration, with n=6 animals allocated per treatment group. Treatment with JNJ-63576253 at 10, 30 or 50 mg/kg, po, qd was initiated 12 days post-castration for a total of 10 days. The vehicle control group for androgen agonism was dosed with combined vehicles (20% HPβCD plus corn oil), whereas a control group for androgen antagonism (inhibition of testosterone propionate (TP)-stimulated ASO growth) received TP + 20% HPβCD. Animal body weights were recorded throughout the study (a) and, at necropsy, the weights of male reproductive tract organs were collected. (b) Exposure to antiandrogens inhibited TP-induced growth of the testes, sex accessory tissues and levator ani-bulbocavernosus (LABC) muscles in a dose-related manner. For androgen antagonism, JNJ-63576253 with co-administered TP group is compared to the reference androgen group (TP), and a statistically significant decrease (*) in tissue weight is considered a positive antagonist result. (c) Blood/ plasma was collected by retro-orbital sampling on study day 0 (pre-dose), day 4 or 5 (1-2 hours post dose) and day 11 (24 hours post last dose) as part of the necropsy for three animals per cohort (n=6/ group).
Figure 6: Castrated male SHO mice were implanted sc on the right flank with human (a) LNCaP AR or (b) LNCaP AR F877L mutant tumor spheroids. After ~45 days post implant, when tumors were established, mice were randomized into experimental groups. The following day, mice were administered with JNJ-63576253 at 30 or 50 mg/kg, po, or enzalutamidte at 30 mg/kg, qd for ~3 weeks (n=9-10/group). Tumors were measured twice weekly and the results presented as the average tumor volume, expressed in mm$^3$ ± SEM of each group. Tumor growth inhibition (TGI) is statistically significant ($p< 0.01$) using a 1-way ANOVA with multiple comparisons Dunnet’s multiple comparisons post-test using Graph Pad Prism software (version 6). (c, d, e) At end of the study, castrated male SHO mice were given a final oral administered of JNJ-63576253 at 30 or 50 mg/kg and terminal plasma and tumor PK was collected at 2, 4, 7 or 24 hours (n = 2-3/timepoint). Data are expressed in mean ± SEM of each group.
Figure 1

(a)

(b)

<table>
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(c)
Figure 3

(a) and (b) Graphs showing cell number as a function of compound concentration for Enzalutamide and JNJ-63576253.

(c) Additional graph with similar data presentation.
Figure 5

(a) Organ Weight Gain (% of TP, ±% CV)
(b) Plasma concentration (ng/ml)
(c) Mean Body Weight (g) ± SD
Molecular Cancer Therapeutics

Discovery of JNJ-63576253, a next-generation androgen receptor antagonist active against wild-type and clinically-relevant ligand binding domain mutations in mCRPC


Mol Cancer Ther Published OnlineFirst March 1, 2021.

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