A clinically relevant androgen receptor mutation confers resistance to 2nd generation anti-androgens enzalutamide and ARN-509

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**Abstract**

Despite the impressive clinical activity of 2nd generation anti-androgens enzalutamide and ARN-509 in prostate cancer patients, acquired resistance invariably emerges. To identify the molecular mechanisms underlying acquired resistance, we developed and characterized cell lines resistant to ARN-509 and enzalutamide. In a subset of cell lines, ARN-509 and enzalutamide exhibit agonist activity, due to a missense mutation (F876L) in the ligand binding domain of the androgen receptor (AR). AR F876L is sufficient to confer resistance to ARN-509 and enzalutamide in in vitro and in vivo models of CRPC. Importantly, the AR F876L mutant is detected in plasma DNA from ARN-509 treated patients with progressive CRPC. Thus, selective outgrowth of AR F876L is a clinically relevant mechanism of 2nd generation anti-androgen resistance that can potentially be targeted with next generation anti-androgens.

**Significance**

A missense mutation in the ligand-binding domain of the androgen receptor, F876L, confers resistance to the 2nd generation anti-androgens enzalutamide and ARN-509 in preclinical models of AR function and prostate cancer and is detected in plasma DNA from ARN-509 treated patients with progressive disease. These results chart a new path for the discovery and development of next generation anti-androgens that could be coupled with a blood-based companion diagnostic to guide treatment decisions.
Introduction

Molecularly targeted cancer therapies have transformed patient care across an increasingly wide range of oncology indications. These agents target key nodal points in tumor cell signaling that regulate the hallmarks of cancer capabilities. However, in the face of these scientific and clinical breakthroughs, a subset of tumors within a given type present with intrinsic resistance and those that do respond will invariably develop acquired resistance. Emergence of acquired resistance via mutation of the therapeutic target is perhaps best understood in imatinib treated CML patients (1). Well studied abl kinase mutations render leukemia cells resistant to imatinib and have spurred the development of multiple next generation abl inhibitors with activity in this setting (2). Importantly, the activity of these 2nd and 3rd generation inhibitors indicate that CML remains “addicted” to deregulated abl kinase, establishing the paradigm of sequential therapy targeting the same driver oncogene in distinct resistant states.

Similar to CML’s dependence upon the abl kinase, accumulating evidence indicates that castration resistant prostate cancer (CRPC) remains dependent upon androgen receptor (AR) signaling (3). A predominant mechanism underlying acquired resistance to 1st generation anti-androgens is an upregulation of AR protein levels. However, mutations in the ligand binding domain of AR have been described in 10 to 20 percent of patients who have progressed on the 1st generation anti-androgen therapies (4, 5). These mutations often confer agonist activity to these agents in a ligand specific manner; T877A or W741C, for example, conferring resistance to flutamide or bicalutamide, respectively (6, 7). The 2nd generation anti-androgens enzalutamide and ARN-509 (8, 9) exhibit full antagonist activity in the context of these mutants in addition to overexpression of wild-type AR, a key determinant of the castration resistant state and acquired resistance to the 1st generation anti-androgens in the absence of AR mutation (10).
demonstrable clinical activity of enzalutamide (recently approved for the treatment of CRPC following treatment with docetaxel; (11)) and ARN-509 (12) underscores the continued role of AR in driving late stage disease. However, the overwhelming majority of ARN-509 or enzalutamide treated patients that exhibit robust declines in PSA eventually develop progressive disease. The molecular underpinnings of acquired resistance to enzalutamide or ARN-509 have yet to be elucidated. To gain insight into the mechanisms of acquired resistance we selected enzalutamide and ARN-509 resistant variants of the LNCaP or LNCaP/AR (overexpressing wild-type AR 3-5 fold above endogenous AR) cell lines through chronic exposure to these agents in vitro. In three independent resistant isolates (2 LNCaP and 1 LNCaP/AR), we identified a novel missense mutation in the AR ligand binding domain (F876L) that confers agonistic activity to both of these agents. Importantly, we find mutations encoding this same amino acid change in ARN-509 treated CRPC patients with progressive disease, indicating that F876L is a clinically relevant mutation that is sufficient to convey resistance to 2nd generation antagonists.
Results

Identification of AR F876L in 2nd generation anti-androgen resistant cell lines

To determine potential mechanisms underlying acquired 2nd generation anti-androgen resistance we developed cell lines resistant to ARN-509 and enzalutamide. Through prolonged in vitro selection, we generated 6 prostate cancer cell lines resistant to ARN-509 (2 each of LNCaP, LNCaP/AR(cs) and LNCaP/AR-Luc) and 4 enzalutamide resistant cell lines (2 each of LNCaP/AR(cs) and LNCaP/AR-Luc). Consistent with published data, in vitro, both ARN-509 and enzalutamide were full proliferative and transcriptional antagonists in all three LNCaP parental cell lines (8, 9). However, in two LNCaP lines selected in ARN-509 (LNCaP ARN-509r1 and 2) and one LNCaP/AR-Luc line selected in the presence of enzalutamide (LNCaP/AR-Luc ENZr2), both ARN-509 and enzalutamide stimulated cell proliferation and transcription on a subset of the 15 AR target genes analyzed (Figure 1A and 1B and Supplementary Figure S1). The partial agonist activity was independent of the compound utilized for selection; ARN-509 and enzalutamide displayed partial agonist activity in all three cell lines regardless of the compound used to derive the resistance variants. Consistent with proliferative activity, ARN-509 or enzalutamide only partially antagonized androgen dependent growth of these resistant lines (Supplementary Figure S1). The conversion of enzalutamide and ARN-509 to partial agonists in these cell lines is unlikely due to AR overexpression as these three cell lines expressed AR at levels approximating or lower than the parental cell line (Supplementary Figure S2).

Given the phenotypic similarities between these three ARN-509/enzalutamide resistant cell lines and prostate cell lines expressing ligand-specific resistance mutations in AR (L701H, W741C, H874L, T877A etc.) we hypothesized that a novel AR mutation(s) may be the resistance driver
Therefore, we sequenced the AR ligand binding domain (c.2013-2757) from the cell lines in which enzalutamide and ARN-509 demonstrate partial agonist activity. In all three cell lines we identified the identical missense mutation of thymine to cytosine at position 2626 of the AR mRNA resulting in the conversion phenylalanine 876 to leucine (Figures 1C, 1D and 1E). Additionally, sequencing of individual subcloned PCR products from the LNCaP/AR-Luc ENZr2 cell line indicated that in all cell lines the F876L mutation arose in the endogenous, T877A containing, AR allele. The identification of the identical mutation in the 3 2nd generation anti-androgen resistant cell lines supports the hypothesis that non-steroidal anti-androgen treatment selects for compound-specific gain of function AR mutations not observed at a high frequency in the untreated population (15) and suggests that the F876L mutation is responsible for the partial agonist activity of ARN-509 and enzalutamide observed in the resistant cell lines. Transcriptional reporter based studies comparing the activities of an AR mutant harboring the single point F876L or the F876L/T877A double mutant found in the in vitro selected lines with wild-type AR or other mutations commonly found in CRPC patients confirmed that mutation of AR F876 to L is sufficient to confer agonist activity to ARN-509 and enzalutamide (Figures 2A, 2B and Supplementary Figures S3 and S4).

While the AR-F876L mutation resides in a hotspot for CRPC AR mutations, this particular mutation has not been reported in prostate cancer or androgen insensitive populations. F876 is located in helix 11 of the AR ligand binding domain and contributes to a small hydrophobic core formed by residues in helix 11 (F786, L880), the loop between helices 11 and 12 (F891) and helix 3 (F697, L701) (Supplementary Figure S5). Unlike the neighboring residue T877, which coordinates hydrogen bonding to the 17β-OH group of dihydrotestosterone, F876 more likely contributes indirectly to binding of the ligand by influencing ligand-induced conformational
changes in helix 12. By affecting protein interactions or the N-C terminal interaction mediated by helix 12, the F876L mutation might affect the off-rate of certain ligand binding interactions as well as the response of AR to these ligands. Consistent with these predictions, in equilibrium AR binding assays, ARN-509 and enzalutamide bound to the F876A mutant with 30 and 48-fold higher affinity than to wild type AR, respectively (Supplementary Figure S6 and Table S1).

**F876L confers ligand selective resistance in vitro**

To further characterize the mechanism of action of the AR-F876L mutant we engineered two LNCaP cell lines to overexpress AR-F876L at levels comparable to the LNCaP/AR(cs) model (Supplementary Figure S7). ARN-509 and enzalutamide had little transcriptional or proliferative activity in LNCaP/AR(cs) and blocked R1881 induced transcription and proliferation to levels consistent with their agonist activity at the highest concentration (Figures 3A and 3B and Supplementary Figure S8). In contrast both enzalutamide and ARN-509 demonstrated robust transcriptional and proliferative agonist activity in F876L-AR expressing cells (Figures 3A and 3B and Supplementary Figures S8 and S9).

Transcriptional activation of androgen regulated AR target genes requires agonist induced DNA binding and subsequent recruitment of transcriptional coregulators (16). To confirm the VP16-AR reporter results indicating ARN-509 and enzalutamide stimulate AR-F876L DNA binding, we performed chromatin immunoprecipitation (ChIP) analysis of 6 AR target genes from cells treated with R1881 and/or each antagonist. In the LNCaP/AR(cs) cells R1881 promoted AR DNA binding (Figure 3C). Consistent with the VP16-AR reporter data, both ARN-509 and enzalutamide promoted AR DNA binding in the LNCaP/SRαF876L cells. In the presence of
R1881 all antagonists inhibited R1881 stimulated AR DNA binding to levels consistent with
their partial agonist or antagonist activity in both cell lines (Supplementary Figure S10).

Many AR full and partial agonists stimulate an interaction between a hydrophobic motif in the
AR amino terminal domain and AF-2, a protein interaction surface formed by helix 12 in the AR
C-terminal ligand binding domain (N-C interaction; (17)). Consistent with the proposed
regulation of ligand-stimulated changes in H12 conformation by the F876L mutation,
enzalutamide and ARN-509 promoted the N-C interaction of AR-F876L but not wild-type AR
(Figure 3D). Thus, the agonist activity of ARN-509 and enzalutamide on AR-F876L appears to
be associated with an agonist-like AF-2 conformation.

**F876L conveys resistance to 2nd generation anti-androgens in vivo**

To determine whether the F876L alteration conveys resistance to enzalutamide and ARN-509 in
vivo, LNCaP cell lines stably expressing AR-F876L were injected (s.c.) into castrated immune-
deficient mice (SCID Hairless Outbred; SHO) and tumors established. Consistent with the in
vitro data, neither ARN-509 nor enzalutamide 30 mg/kg/day impacted the growth of
LNCaP/SRαF876L tumors (Figure 4). This lack of activity was not a function of unexpectedly
low compound exposure as day 28 plasma drug levels were quantified and were consistent with
previous LNCaP/AR xenograft studies ((9); Supplementary Table S2). In addition, in a parallel
experiment, ARN-509 30 mg/kg/day exhibited robust anti-tumor activity in the LNCaP/AR(cs)
model, consistent with previous results ((9); Figure 4).
**F876L encoding mutations are found in ctDNA from ARN-509 treated patients with progressive disease**

To determine whether the F876L mutation is a likely determinant of acquired resistance to 2nd generation anti-androgens in CRPC patients, we analyzed circulating tumor DNA (ctDNA) in plasma from patients who participated in the Phase I portion of a clinical trial of ARN-509 using the sensitive, emulsion PCR-based BEAMing method (18). BEAMing has been successfully used to detect a variety of tumor derived mutations in driver oncogenes such as PIKC3a and K-ras in ctDNA derived from human plasma (19). The Phase I study was a first-in-man dose escalation study that assessed safety, tolerability, pharmacokinetics, pharmacodynamics, and antitumor activity of ARN-509 in men with metastatic CRPC across 9 dose levels (12). Figure 5A depicts the PSA response (% change from baseline) for each individual patient. At 12 weeks, 46.7% of patients had ≥ 50% decline in PSA as compared to baseline. Pre-treatment and during treatment plasma samples were analyzed. Time of BEAMing analysis is indicated by the terminal end of the PSA response line. Eighteen out of the 29 patients had PSA above baseline at time of analysis indicating either intrinsic or acquired resistance to ARN-509.

Three probes were designed to monitor the 3 nucleotide changes that can encode for the F876L amino acid substitution (Supplementary Table S3). Dilution mixing experiments with the mutant sequence and wild-type DNA indicated a technical sensitivity of 0.02% (potential to detect 1 mutant sequence among 5000 wild-type). In an initial screen of plasma samples from the 29 ARN-509 treated patients, evidence of the mutation was detected in 3 patients (Table 1 and Supplementary Table S4). At time of BEAMing analysis, Patient 7 and 10 had PSA levels above
baseline, whereas Patient 13 has evidence of rising PSA above the treatment nadir (Supplementary Figure S11). In all 3 patients, the nucleotide change c2628a was detected. In one of these 3 patients (Patient 10) the t2626c mutant was also detected, indicative of polyclonal disease. F876L encoding mutations were not detected in any of pre-treatment samples (0/29) suggesting that if present prior to ARN-509 treatment, they were below the limit of detection or the mutations arose de novo during ARN-509 treatment. In either scenario, the data support the hypothesis that the selective outgrowth of lesions bearing the mutant allele to levels sufficient to detect in ctDNA is dependent on chronic exposure to ARN-509 and is associated with rising PSA. To further establish the association of F876L with progressive disease, we analyzed plasma samples taken at additional timepoints from the 3 patients scored positive during the initial screen (Table 1). In patient 10, the mutation was not detected at the one other timepoint analyzed (Cycle 4; PSA 102% of baseline). In Patient 13, the mutation was not detected at Cycle 4 (PSA 16.2% of baseline) or at Cycle 12 (PSA 39.4% of baseline; patient was scored positive at Cycle 11). The mutant sequence at Cycle 11 was at the limit of detection and would be estimated to arise via amplification of a single mutant molecule. Although PSA of Patient 13 was slowly rising from the treatment nadir at Cycles 11 and 12, at both time points PSA was still >60% below study start, and thus frank resistance had not yet emerged. Identification of the mutant sequence at the limit of detection likely reflects presence of a relatively rare, mutant clone that has potential to expand under continued selective pressure and eventually drive progressive disease. Alternatively, the correlation between F876L detection and PSA levels may be compromised by the use of plasma preparation protocols that have not been optimized to enrich for ctDNA and thus the analyzed samples may contain variable levels of contaminating lymphocyte DNA that could diminish the tumor-derived signal (Materials and Methods).
Given the relatively long duration of treatment of Patient 7, plasma from selected time points collected while the patient was responding to treatment (> 90% decline from baseline; Cycle 4, 8 and 10) or starting to exhibit progression (Cycle 15 and 19) (Figure 5B) were analyzed. Interestingly, mutations were not detected in the 3 samples from treatment Cycles 4, 8 and 10 whereas the c2628a mutation was detected in the 2 samples analyzed from Cycles 15 and 19. These clinical data are consistent with the preclinical data indicating that the F876L amino acid change is sufficient to convey resistance to ARN-509.
Discussion

The clinical activity of 1st and 2nd generation hormonal therapy in both castration sensitive and resistant prostate cancer underscores the central role of the androgen receptor across the full spectrum of prostate cancer disease states. Understanding the mechanisms of acquired resistance to 1st generation anti-androgens (10) was the first step towards the discovery and development of two 2nd generation agents, enzalutamide and ARN-509 (8, 9). It follows that understanding mechanisms of acquired resistance to the 2nd generation anti-androgens will inform design, discovery and development of next generation anti-androgens with broad activity across multiple resistant states. In both pre-clinical functional studies and a correlative clinical study, we describe an amino acid change F876L as one mechanism (of undoubtedly of multiple) of acquired resistance to both enzalutamide and ARN-509.

The discovery of a novel AR mutation that confers resistance to both enzalutamide and ARN-509 underscores the ligand selective nature of such mutations and indicates that late stage CRPC bearing such mutations are still dependent on AR signaling for growth and survival. Based on the hypothesis that gain of function mutations could contribute to acquired 2nd generation anti-androgen resistance Balbas et al. (20) recently described a forward genetic screen for AR mutations that confer agonist activity to enzalutamide. Consistent with our results, F876 was the only amino acid residue identified in the screen capable of inducing an antagonist to agonist switch. These results suggest that it may be possible to therapeutically target the majority of 2nd generation anti-androgen resistance arising through AR mutation by developing AR antagonists that function in the context of AR (wild-type) overexpression, as well as the F876L mutation. Balbas et al. used a rational drug design approach to identify the novel thiohydantoin-based F876L AR antagonist, DR103 which was engineered to accommodate structural changes.
introduced by the F876 mutation. DR103 stands as the first published proof of concept of the approach to target AR F876L in the setting of acquired resistance to ARN-509 and enzalutamide

Using the BEAMing technique on ctDNA from patient plasma, mutations encoding F876L were detected in 3/29 patients who had received the 2\textsuperscript{nd} generation anti-androgen ARN-509 as part of a Phase I dose escalation study and had ctDNA samples analyzed. Across doses ranging from 30 to 480 mg per day, 14/30 patients exhibited $\geq 50\%$ reduction of PSA at 12 weeks (12). Consistent with the F876L mutation as a driver of acquired resistance to ARN-509, the mutation was detected in 0/29 pre-treatment samples. Two of the mutant bearing patients had frank PSA elevation whereas the PSA of the 3\textsuperscript{rd} mutant bearing patient was rising from treatment nadir at the time of BEAMing analysis. Given the wide range in dose, heterogeneity with respect to intrinsic resistance and therefore duration of treatment (selective pressure) of the patients screened it is difficult to estimate the prevalence of the F876L encoding mutations in patients that have progressed on 2\textsuperscript{nd} generation anti-androgens. While this initial study was limited to screening of ARN-509 treated patients, based on our preclinical work and the work of Balbas et al, F876L encoding mutations are predicted to arise in enzalutamide treated patients with progressive disease with similar frequency and clinical impact. A more accurate delineation of the frequency of F876L in patients who develop progressive disease while treated with 2\textsuperscript{nd} generation anti-androgens will require a larger study of patients, preferably in early stage of CRPC such as pre-chemotherapy or non-metastatic CRPC where intrinsic resistance will be minimal and treatment duration relatively long (21).
While the BEAMing is a very sensitive technique to detect mutations in ctDNA, it only probes for *a priori* defined changes. It is possible that additional resistance-conferring mutations arise in enzalutamide and ARN-509 treated patients that were not defined in the preclinical studies and that with the use of emergent ctDNA-based Next Generation Sequencing (NGS) approaches other genetic changes will be uncovered. These data are essential to the development of next generation anti-androgens that target the broadest spectrum of resistance conferring mutations.

The identification of novel amino acid change that bestows resistance to the 2\textsuperscript{nd} generation anti-androgens enzalutamide and ARN-509 enables the design and or discovery of next generation agents. Given the multi-focal and thus potentially polyclonal nature of CRPC, next generation agents must have potent activity on both wild-type and mutant AR along with good pharmacokinetics to ensure full receptor occupancy and AR selectivity to minimize undesirable off-target effects \((9, 22)\). The real-time clinical identification of F876L (and potentially other mutations) in patients who have progressed on 2\textsuperscript{nd} generation anti-androgens using a non-invasive ctDNA-based companion diagnostic would distinguish patients most likely to benefit from next generation anti-androgen therapy and guide treatment decisions.
Materials and Methods

Cell culture and reagents

LNCaP (ATCC, purchased 2009), LNCaP/AR(cs) (a LNCaP cell line engineered to overexpress wild-type AR 3- to 5-fold higher than the parental LNCaP cell line, gift from Charles Sawyers, 2009) (23) and LNCaP/AR-Luc (a LNCaP/AR(cs) cell line containing a stable integrated androgen responsive ARR2-Pb-luc reporter, gift from Charles Sawyers, 2009) (24), and CV1 (ATCC, purchased 2010) cells were maintained in RPMI 1640 supplemented with 10% FBS (Hyclone). HepG2 cells were obtained from ATCC in 2009 and maintained in MEM plus 10% FBS. The cell lines used have not been further authenticated. All media also contained 1mM Na Pyruvate and 1X Non-essential amino acids. Unless otherwise noted all tissue culture medium was purchased from Mediatech.

DNA and plasmids and are detailed in the Supplementary Materials and Methods.

ARN-509 and enzalutamide were synthesized at Aragon Pharmaceuticals. Unless otherwise indicated all chemicals were purchased from Sigma-Aldrich.

Resistant line selection

LNCaP, LNCaP/AR(cs) or LNCaP/AR(cs)-Luc cells were cultured in increasing concentrations of either ARN-509 or enzalutamide (800 nM to 6 μM) over a course of 6 months. After selection the cells were maintained in RPMI plus 10% FBS and 6 μM ARN-509 or enzalutamide.

Stable cell line generation

Detailed protocol used to generate the PC3 and LNCaP cell lines stably expressing AR and AR-F876L are provided in the Supplementary Materials and Methods.
Whole-cell extract binding assay

Binding assays were performed as described in (9) using PC3 cells stably expressing wild-type human AR or AR-F876L. $K_i$ was calculated according to Cheng-Prusoff (25) as $K_i = IC_{50}/(1 + ([^3]H-R1881)/K_d))$, $[^3]H-R1881 = 0.6$ nM.

Proliferation assays
Details of the proliferation assays are provided in the Supplementary Materials and Methods.

Transcriptional reporter assays

Transcriptional reporter assays were performed as described in (9). Specific details of the individual assays are presented in the Supplementary Materials and Methods.

RNA isolation and real-time PCR (RT-PCR)

RNA isolation and qPCR were performed as described in Clegg et al (9). Expression data was log2 standardized and graphed in cell plot using JMP8 software (SAS). Real-time PCR primer sequences are listed in Supplementary Table S5.

Chromatin immunoprecipitation assay

ChIP assays were performed as described previously (26). Specific details are presented in Supplementary Materials and Methods. Real-time PCR primer sequences used in the ChIP assays are listed in Supplementary Table S6.

In vivo pharmacology

All animal studies performed at Aragon Pharmaceuticals Inc. and were carried out under protocols approved by the Institutional Animal Care and Use Committee and institutional guidelines for the proper, humane use of animals in research were followed. In vivo xenograft
experiments were carried out in SCID Hairless Outbred (SHO) male mice (Charles River Laboratories). Mice were orchiectomized under isoflurane anesthesia and were given 7-10 days to recover. LNCaP/AR(cs) or LNCaP/SRαF876L cells were suspended in 50% RPMI, 50% Matrigel (BD Biosciences), and $3 \times 10^6$ cells/xenograft were injected in a volume of 100 μL. Animals were observed weekly until tumor growth was apparent. After 40-60 days post-injection, animals were randomized into cohorts with equivalent mean tumor burden and range (150-250 mm$^3$). All compounds were administered daily by oral gavage. For all LNCaP/AR(cs) xenograft studies ARN-509 and enzalutamide drug stocks were prepared in 18% PEG-400, 1% Tween-80 and 1% povidone, and were formulated for dosing in 15% Vitamin E-TPGS and 65% of a 0.5% w/v CMC solution in 20 mM citrate buffer (pH 4.0). ARN-509 and enzalutamide pharmacokinetics were assessed at the end of study as described previously (9).

**BEAMing assay**

All BEAMing assays were performed by Inostics GmbH (Hamburg, Germany). Plasma was collected using a standard clinical laboratory method. Briefly, blood was collected in K2-EDTA evacuated tubes and mix thoroughly by slowly inverting several times. Within 30 minutes of collection tubes were spun at 2000 x g for 15 minutes. Plasma was decanted, transferred to cryo storage tubes. Within 90 minutes of decantation, plasma was stored at -70°C or lower until analysis. DNA was purified from 300-500 μl plasma aliquots and extracted as previously described (19). Mutation detection was performed according to BEAMing technology as previously described (19). Briefly, in the initial PCR step, the target region (~100 bp) was amplified using gene-specific primers with tag sequences and subjected to an emulsion PCR.
containing primer coated magnetic beads. After emulsion PCR discrimination of wild-type and mutant beads was performed by allele-specific hybridization followed flow cytometry. Flow cytometry results were analyzed using FCS Express (De Novo Software, Los Angeles, CA) resulting in the quantification of the ratio of the mutant allele over the wild type alleles.
Acknowledgments

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References


Table 1. BEAMing Results from F876L Positive Patients

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*Treatment cycle is 4 weeks; Cycle 0 is pre-treatment timepoint; # See Methods for details around assay sensitivity and sample scoring positive.
**Figure Legends**

**Figure 1.** ARN-509 and enzalutamide resistance. (A) LNCaP and LNCaP ARN-509r1 cell proliferation. LNCaP and LNCaP ARN-509r1 cells were cultured in the presence of hormone depleted medium for 2 days followed by ligand addition. Proliferation is quantified by CellTiter-Glo luminescence based viability assay after 7 day compound treatment. (B) qPCR analysis of AR-regulated gene expression. LNCaP, LNCaP/AR(cs), LNCaP/AR-Luciferase and three 2nd generation anti-androgen resistant cell lines were cultured for 3 days in hormone depleted medium followed by treatment with vehicle, 1 nM R1881 or 30 μM compound. Gene expression was normalized to GAPDH. (C) Sanger sequence identification of F876L mutation. DNA sequence chromatograms obtained from direct sequencing of AR-LBD PCR products from three ARN-509 and enzalutamide gain of function resistant cell lines. Sequence analysis demonstrates the presence of heterogeneous c.2626 T > C (encoding F876L) mutation in cell lines demonstrating ARN-509 and enzalutamide gain of function activity. (D) AR amino acid and protein sequence indicating F876L and T877A AR mutations. (E) Schematic representation of AR domain structure showing amino acids that when mutated display altered ligand activity in CRPC.

**Figure 2.** ARN-509 and enzalutamide are partial agonists of AR F876L. (A) Transcriptional agonist and antagonist activity ARN-509 and enzalutamide on wild-type or F876L AR. Transcriptional activation of a 4X ARE-luciferase reporter was measured in the presence of increasing compound concentration in the absence or presence of 1 nM R1881 (for wild-type AR) or 5 nM R1881 (for F876L AR). (B) VP16-AR and F876L VP16-AR agonist and antagonist activity of ARN-509 and enzalutamide. 4X ARE-luciferase reporter activity was monitored in the presence of increasing compound concentration in the absence or presence of
90 pM R1881 (for wild-type VP16-AR) or 1 nM R1881 (for F876L VP16-AR). Expression
levels for wild-type and F876L AR are presented in Supplementary Figure S3.

**Figure 3.** F876L AR mutation confers partial agonist activity to ARN-509 and enzalutamide.

(A) qPCR analysis of AR-regulated gene expression. LNCaP/AR(cs) and LNCaP SRαF876L
were cultured for 3 days in hormone depleted medium followed by treatment with vehicle, 1 nM
R1881 and 1, 3, 10 and 30 μM compound in the presence or absence on 1nM R1881. (B)
LNCaP/AR(cs) and LNCaP/SRαF876L cell proliferation. Cells were cultured in the presence of
hormone depleted medium for 2 days followed by ligand treatment for 7 days. Proliferation is
quantified by CellTiter-Glo luminescence based viability assay. (D) Chromatin
immunoprecipitation assay of AR. ChIP assays were performed on LNCaP/AR(cs) and LNCaP
SRαF876L cells incubated for 3 days in hormone depleted medium followed by 4 hour ligand
treatment. (C) AR N/C interaction assay. Ligand induced N/C interaction was monitored via
mammalian two hybrid assay in HepG2 cells. Antagonists were assayed at 8 μM, R1881 at
1nM.

**Figure 4.** AR F876L mutation confers ARN-509 and enzalutamide resistance in vivo.

LNCaP/AR(cs) and LNCaP/SRαF876L tumor xenografts. Castrate male mice bearing tumors
were treated daily with vehicle or 30 mg/kg/day compound. Tumor growth for each group is
presented as the average tumor volume ± SEM.

**Figure 5.** Identification of AR-F876L in ARN-509 treated patients. (A) PSA response of 29
patients analyzed for F876L mutation. Terminal end of PSA response line is time at which the
patient plasma was screened for F876L mutation using BEAMing analysis. The plasma used in
this study was initially collected to determine pharmacokinetics of ARN-509 and as such the samples were not prepared using methodology to maximize ratio of ctDNA to lymphocyte DNA. Treatment cycle is 4 weeks. (B) PSA response of patient positive for F876L. PSA response of patient 7 at indicated treatment cycle. Circulating plasma was analyzed for the F876L at times indicated with arrows. The plasma samples with no detectable mutant are notated as “w.t.”, the presence of the F876L mutation is represented by “m”. A plasma sample was called positive for the mutant if the percent of mutant beads was above the cut-off (0.02%) and the number of mutant copies estimated to be ≥ 0.5 (number of genome equivalents in the plasma sample x mutant bead fraction= ≥ 0.5).
Figure 1

A. LNCaP LNCaP ARN-509r1

Relative Luciferase Activity

Concentration (Log M)

B. LNCaP LNCaP/AR(cs) LNCaP/AR-Luc LNCaP ARN-509r1 LNCaP ARN-509r2 LNCaP/AR-Luc

Fold DMSO (Log2)

Vehicle R1881 Enzalutamide ARN-509

C. LNCaP ARN-509r1 LNCaP ARN-509r2 LNCaP/AR-Luc LNCaP/AR-Luc

2nd Generation Anti-androgen Stimulated Non-stimulated

D. gcgagagagctgcctcactttaacttttgacctgctaatacg

E. 1 NTD DBD LBD

L701 W741 H874 T877 F876919
Figure 2

A. WT

B. VP16-WT

Transcriptional Activity (Percent Maximum)

Concentration (Log M)

F876L

DNA Binding Activity (Percent Maximum)

Concentration (Log M)

VP16-F876L
Figure 3

A. LNCaP/AR(cs) vs LNCaP/SRαF876L

B. Concentration response curves for LNCaP/AR(cs) and LNCaP/SRαF876L

C. Gene expression analysis of LNCaP/AR(cs) and LNCaP/SRαF876L

D. Fold induction for Wild-type and F876L
Figure 4

LNCaP/AR(cs) vs. LNCaP/αF876L

Tumor Volume (mm$^3$) vs. Treatment Day

- Vehicle
- ARN-509 30mg/kg
- Enzalutamide 30mg/kg
Figure 5
A clinically relevant androgen receptor mutation confers resistance to 2nd generation anti-androgens enzalutamide and ARN-509

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