Supplemental Materials and Methods

Discovery and development of ARN-509, a novel anti-androgen for the treatment of prostate cancer

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Materials and Methods

All experimental procedures using commercially available reagents did so according to manufacturers’ instructions or published protocols.

Chemicals

Bicalutamide (Waterstone Technology or Investigational Drug Pharmacy) and R1881 (NEN Life Sciences; PerkinElmer Life and Analytical Sciences) were obtained commercially.
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All other anti-androgens were synthesized at Aragon or MSKCC. $^{18}$F-FDHT was synthesized at MSKCC. Stock and serial dilutions were in dimethyl sulfoxide (DMSO) unless otherwise specified.

**Cell-lines**

LNCaP (ATCC), LNCaP/AR, LNCaP/AR(cs) (1), LNCaP/AR-luc (2), LAPC4 (3), VCaP (ATCC) and PC-3 cells were obtained and propagated as previously described (4) in either RPMI 1640, DMEM-HG or Iscove’s media supplemented with 10% FBS (Omega Scientific). HepG2 cells were maintained in RPMI 1640 supplemented with 10% FCS.

**Ligand binding studies**

**Whole cell LNCaP/AR:** Whole-cell competitive binding assays were performed in LNCaP/AR(codon-switch) (LNCaP/AR(cs)) (harbors a mixture of exogenous wild-type AR and endogenous mutant AR (T877A)) and cells propagated in Iscove’s or RPMI media supplemented with 10% fetal bovine serum (FBS, Omega Scientific), or during the assay with 10% charcoal-stripped, dextran-treated fetal bovine serum (CSS). Cells were pre-incubated with $^{18}$F-FDHT, increasing concentrations (1pM to 1µM) of cold competitor were added, and the assay was performed according to published procedures to measure specific uptake of $^{18}$F-FDHT (4). IC$_{50}$ values were determined using a one site binding model with least squares curve fitting (Origin, OriginLab, Northampton, MA) and $R^2 > 0.99$.

**Whole-cell extract MDA-MB-453 cells:** MDA-MB-453 cells (endogenous wild-type AR; ATCC: HTB131) were cultured in RPMI 1640 containing 20 mM HEPES, 4 mM L-glutamine, 10 µg/mL human insulin, 10% FBS and 20 µg/mL gentamicin. After reaching 90% confluence, cells were harvested, resuspended in TEGM (10 mM Tris-HCl pH 7.2, 1 mM EDTA, 10% glycerol, 1 mM β-mercaptoethanol, 10 mM sodium molybdate), and frozen in liquid
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nitrogen in 10 mL aliquots containing $4 \times 10^7$ cells/mL. Binding reactions (60 μL) were carried out in 96-well plates in TEGM, and typically contained 24 μL cell lysate, 1.2 nM $^3$H-R1881 (Perkin Elmer), and $10^{-10}-10^{-4}$ M of the respective competitive ligand. Reactions were incubated at 4 °C overnight. Bound and unbound ligands were separated by ultrafiltration using a Unifilter-96 GF/C filter plate (Perkin Elmer). Bound $^3$H-R1881 was eluted in 30 μL/well Microscint-20 and quantified using a Top Count. $K_i$ was calculated according to Cheng-Prusoff (5) as $K_i = IC_{50}/(1 + ([^3H-R1881]/K_d))$.

**In vitro:** Competitor assay kits (green, Invitrogen) were used according to published procedures (4) to determine relative *in vitro* binding affinities of ARN-509 for the rat AR ligand binding domain (LBD), human progesterone receptor (PR) LBD, and full-length human estrogen receptor-alpha (ERα) and human glucocorticoid receptor (GR). Each hormone dose was performed in triplicate, relative error was calculated from the standard error of the mean (SEM), and binding curves were fit using a single binding site competition model (Prism statistical analysis software package) with $R^2 > 0.8$. Experiments were conducted multiple times with SEM < 0.3 log units from the average logIC$_{50}$ value. $K_i$ values were calculated as averages across experiments with SEM, and binding affinities were reported as a percentage relative to the tight-binding ligand control for that receptor.

**Proliferation assays**

Trypsinized VCaP cells were adjusted to a concentration of 100,000 cells per mL in phenol-red-free RPMI 1640 (with 5% CSS), and dispensed in 16 μL aliquots into CellBIND 384 well plates (Corning, Lowell, MA). Cells were incubated for 48 hours, after which ligand was added in a 16 μL volume to the RPMI culture medium. For the antagonist mode assay, the ligands were diluted in culture medium also containing 30 pM R1881 (final [R1881] = 15
After 7 days’ incubation, 16 µL of CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI) was added and Relative Luminescence Units (RLUs) measured. In the agonist mode assay, percent viability of the samples was calculated as: % viability=[RLU sample-RLU medium without cells]/[RLU DMSO treated cells-RLU medium without cells]. In the antagonist mode assay, the percent viability of the samples was calculated as: % viability=[RLU sample-RLU VCaP without R1881]/[RLU R1881-treated cells - RLU VCaP without R1881].

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

LNCaP/AR cells were grown for 3 days in RPMI containing 5% CSS. Cells were then treated for 24 h with ligand and RNA was isolated using the Aurum™ total RNA isolation kit (BIO-RAD, Hercules, CA). RNA (1 µg) was reverse-transcribed using the iScript cDNA synthesis kit (BIO-RAD, Hercules, CA). RT-PCR was performed using the Applied Biosystems 7900HT instrument and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). GAPDH expression was used to normalize all real-time data. Following normalization, the expression data was log2 standardized and clustered with the Ward hierarchical clustering algorithm to produce a gene expression heatmap using JMP8 software (SAS, Cary, NC). Real-time primer sequences are listed in Supplemental Table 1.

**Fluorescence microscopy**

LNCaP cells (10^5 cells/well) were transfected with AR-EYFP as previously described (4), and incubated for 4 hours with AR ligands in hormone-depleted media. Imaging was performed on a Leica TCS AOBS SP2 inverted confocal microscope (MSKCC Molecular Cytology Core Facility).

**Chromatin immunoprecipitation (ChIP)**
LNCaP/AR(cs) cells (10^7 cells/condition) were treated for 1 h with DMSO, R1881 (1nM), and/or anti-androgen (10 μM) in RPMI media supplemented with 5% CSS. Cells were cross-linked using 1% paraformaldehyde (Electron Microscopy Sciences) for 10 minutes, and neutralized by addition of glycine followed by centrifugation (4°C, 4000 rpm, 5 minutes). A chromatin immunoprecipitation assay kit (Upstate) was used according to manufacturer’s protocols, together with an antibody for androgen receptor (PG-21, Upstate). Sonication of lysates for 15 minutes using a Bioruptor (Diagenode) ensured DNA fragment sizes of 500-1000 bp. Amplification of immunoprecipitated DNA by real-time PCR was performed (ABI Power SYBR Green PCR mix) using the following primers: PSA enhancer forward ATGTTCACATTAGTACACCTTGCC, reverse TCTCAGATCCAGGCTTGCTTACTGTC (6); TMPRSS2 enhancer forward TGGTCCTGGATGATAAAAAAAGTTT, reverse GACATAACGCCCCACACACAGA (7).

**Luciferase transcriptional reporter gene assays**

Transcriptional assays were performed by seeding 100 μL of LNCaP/AR-luc cells at a density of 25,000 cells/mL into 96-well cell culture plates in RPMI 1640 supplemented with 10% CSS and ligands added 24 h later. For AR agonist assays, ligands were serially diluted in RPMI 1640 with CSS, and 50 μL was added to the cells. For AR antagonist assays, ligands were serially diluted in RPMI 1640 with CSS and R1881, and 50 μL was added to cells, with a final R1881 concentration of 0.1 nM. Following 40 h incubation the medium was removed and the cells were lysed in 40 μL of lysis buffer (25 mM Tris phosphate, 2 mM CDTA, 10% glycerol, 0.5% triton X-100, 2 mM DTT). Firefly luciferase activity was measured immediately following the addition of 50 μL luciferase buffer (20 mM tricine, 0.1 mM EDTA, 1.07 mM (MgCO₃)_4
Mg(OH)$_2$ • 5H$_2$O, 2.67 mM MgSO$_4$, 33.3 mM DTT, 270 µM co-enzyme A, 470 µM luciferin, 530 µM ATP).

**VP16-AR transcriptional reporter assays**

AR-VP16 DNA binding assays were performed by seeding 100 µL of Hep-G2 cells in RPMI 1640 with 10% CSS into 96-well cell culture plates at a density of 250,000 cells/mL. Cells were transiently transfected in triplicate using Lipofectin (Life Technologies) according to the manufacturer’s protocol with 33.3 ng pACT-AR (8) (gift of Howie Shen and Steven Balk (BIDMC)), 66.7 ng 4XARE-Luc (4), 16.7 ng CMVpRL (normalization vector), and 43.3 ng pCMX (filler DNA). Transfected cells were incubated overnight then treated with ligand. For agonist assays, ligands were serially diluted in RPMI with CSS and 50 µL was added to the cells. For antagonist assays, ligands were serially diluted in RPMI with CSS also containing R1881, and 50 µL was added to the cells to give a final R1881 concentration of 0.1 nM. Following 48 h incubation the media was removed and cells lysed in 40 µL lysis buffer (25 mM Tris phosphate, 2 mM CDTA, 10% glycerol, 0.5% triton X-100, 2 mM DTT). Firefly luciferase activity was measured immediately following addition of 40 µL luciferase buffer (20 mM tricine, 0.1 mM EDTA, 1.07 mM (MgCo$_3$)$_4$ Mg(OH)$_2$ • 5H$_2$O, 2.67 mM MgSO$_4$, 33.3 mM DTT, 270 µM Coenzyme A, 470 µM luciferin, 530 µM ATP). Renilla luciferase was measured following the addition of 40 µL coelenterazine buffer (1.1 M NaCl, 2.2 mM Na$_2$EDTA, 0.22 M K$_3$PO$_4$ (pH 5.1), 0.44 mg/mL BSA, 1.3 mM NaN$_3$, 1.43 µM coelenterazine, final pH adjusted to 5.0).

**In vivo pharmacology**

All animal studies performed at MSKCC, Aragon Pharmaceuticals Inc. and Covance Laboratories Inc. were carried out under protocols approved by the MSKCC or Aragon Institutional Animal Care and Use Committees and institutional guidelines for the proper,
humane use of animals in research were followed. In vivo xenograft experiments to determine anti-tumor response were carried out in SHO SCID male mice (Charles River, Hollister, CA.). Mice were orchiectomized under isoflurane anesthesia and were given 2-3 days to recover prior to tumor cell injection. LNCaP/AR(cs) cells were suspended in 50% RPMI (Hy-Clone), 50% Matrigel (BD), and $5 \times 10^6$ cells/xenograft were injected in a volume of 100 μL. Animals were observed weekly until tumor growth was apparent. From 24 d post-injection, tumors were measured weekly, and after 40-60 days post-injection, animals were randomized into cohorts of equivalent mean (150-250 mm$^3$) and range tumor burden. All compounds were administered daily by oral gavage. Statistical analyses were performed using Graphpad Prism.

For all LNCaP/AR(cs) xenograft studies ARN-509, MDV3100 and bicalutamide 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).

In vivo experiments to determine pharmacodynamic or therapeutic response of tumors derived from LNCaP/AR-luc in castrated male SCID mice were performed as previously described (4), with test compounds formulated in an aqueous slurry of 1% carboxymethyl cellulose (CMC), 0.1% Tween-80, 5% DMSO.

In vivo pharmacodynamic studies

Pharmacodynamic studies with LNCaP/AR-luc xenografts in castrate male SCID mice were performed as previously described (4). Formalin-fixed, paraffin-embedded tissue was processed and stained for hematoxylin and eosin (H&E), terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) or immunohistochemistry (IHC) for Ki67 as previously described (4). In vivo luciferase imaging of mice with LNCaP/AR-luc xenografts was performed
Development of anti-androgen ARN-509 according to published methods (4), and data analyzed using Living Image 2.30 software.

As part of an Investigational New Drug (IND)-enabling toxicity and toxicokinetic study, ARN-509 was administered to male beagle dogs (aged 6 to 7 months, with body weights ranging from 9.3 to 11.2 kg), by Covance Laboratories Inc., in accordance with the United States Food and Drug Administration (FDA) Good Laboratory Practice (GLP) Regulations. ARN-509 was administered daily for 28 days at 0 mg/kg (5 dogs) or 10 mg/kg (4 dogs) by oral gavage (po). ARN-509 (3.33 mg/mL) was formulated as a suspension in labrasol (Gattefosse)(10% v/v), lactic acid (10% v/v) and soybean oil (10% v/v) and brought up to volume with 50mM phosphate buffer. The placebo oral formulation contained 0 mg/mL ARN-509.

On Day 29, the animals were weighed, sacrificed and necropsied by appropriately trained personnel using approved procedures. Organ weights were recorded, organs were fixed in 10% neutral-buffered formalin (prostate) or modified Davidson’s fixative (testis and epididymis) and 4 µm-thick serial tissue-sections of paraffin-embedded tissues were prepared on charged glass slides. The tissue-sections were stained by the MSKCC Laboratory of Comparative Pathology with H&E for histologic analysis, or with TUNEL (9) for analysis of apoptosis, or they were used for immunohistochemistry (IHC) for Ki67 (Vector Laboratories VP-K451), antibody (Ab) dilution 1:10000). Antigen retrieval was performed prior to immunohistochemical staining by microwave irradiation (high power, 15 minutes) in sodium citrate buffer (10 mM, pH 6.0). The Vectastain ABC Elite Kit (Vector Labs # PK-6100) was used for secondary visualization of antigens following labeling with the primary antibody or IgG control. Appropriate positive and negative controls were included. For each animal, one transverse prostate section and two longitudinal epididymis sections (complete) were evaluated by a trained pathologist and microscopy was performed using an Olympus BX45 microscope with images captured using an
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Olympus DP25 camera on a U-TVO.5XC attachment and Olympus DP2-BSW (version 2.2) software. Slides were scanned using the Mirax Scan 150 (Zeiss) with 20x/0.8NA objective and automatic exposure settings, and image files are available online (10).

**Mouse and dog pharmacokinetics**

Mouse (male CD-1, Charles River) or beagle dog (Charles River) plasma samples (25 µL) were combined with 100 µL of acetonitrile:methanol:acetic acid, 1:1:0.001, v/v/v containing 500 ng/mL ARN-509-d3 as an internal standard. Precipitated proteins were removed by centrifugation at 1,500 g for 20 minutes at 5°C. Supernatant (50 µL) was diluted with 400 µL of 2:1 water:acetonitrile. ARN-509 concentrations were quantified using the LC-MS/MS method below.

**Tumor and brain tissue-distribution assays**

Pre-weighed tumor samples were homogenized using a “Fastprep” system (MP Biomedicals Inc., Solon, OH). Tumor samples were combined with 3 volumes of deionized water and zirconium beads (1mm) and processed for up to 4x1 min cycles with cooling on ice between cycles. Tumor homogenate (25 µL) was combined with 100 µL of acetonitrile:methanol:acetic acid, 1:1:0.001, v/v/v containing 50 ng/mL ARN-509-d3 as an internal standard. Precipitated proteins were removed by centrifugation at 1,500 g for 20 minutes at 5°C. Supernatant (75 µL) was diluted with 100 µL of 2:1 water:acetonitrile. ARN-509 or MDV3100 concentrations in tumors and plasma were quantified using the LC-MS/MS method below.

Mouse brains were dissected along the sagittal midline, and one half was weighed, and homogenized using a “Fastprep” system (MP Biomedicals Inc., Solon, OH) with 3 volumes of deionized water and zirconium beads (1mm) for up to 1 minute with cooling on ice. Brain
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Homogenate (25 µL) was combined with 100 µL of acetonitrile:methanol:acetic acid, 1:1:0.001, v/v/v containing 50 ng/mL ARN-509-d3 as an internal standard. Precipitated proteins were removed by centrifugation at 1,500 g for 20 minutes at 5°C. Supernatant (75 µL) was diluted with 125 µL of 2:1 water:acetonitrile. ARN-509 or MDV3100 concentrations in brains and plasma were quantified using the LC-MS/MS method below.

**LC-MS/MS method**

The following method was used for LC-MS/MS analyses:

Injector: (10 µL) Waters Acuity w/ cooled sample organizer (9°C); UPLC: Waters Acuity with column oven (30°C); column: Waters BEH C18 (1.7 µm) 2 x 50 mm; mobile phase: (A) 100% water + 0.1% formic acid (FA); (B) 100% acetonitrile + 0.1% FA; retention times: 1.61 min ARN-509, 1.58 minutes MDV3100. The HPLC gradient program was as follows:

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow Rate (mL/min)</th>
<th>A (%)</th>
<th>B (%)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>0.6</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>0.6</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>0.51</td>
<td>0.6</td>
<td>70</td>
<td>30</td>
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<tr>
<td>0.8</td>
<td>0.6</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>1.5</td>
<td>0.6</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>2</td>
<td>0.6</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>2.1</td>
<td>0.6</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0.6</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

Analytes were detected by MS/MS analysis using an AB Sciex API-4000 triple-quad mass spectrometer (Foster City, CA) in positive ionization mode.

**Assay ranges were as follows:**

<table>
<thead>
<tr>
<th>Assay Ranges (LLOQ – ULOQ) in µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>ARN-509</td>
</tr>
<tr>
<td>MDV3100</td>
</tr>
</tbody>
</table>

LLOQ: Lower limit of quantitation
ULOQ: Upper limit of quantitation
Pharmacokinetic parameters were calculated using non-compartmental analysis (Microsoft excel 2007) of plasma, tumor or brain concentration data (11).

**In vitro serum-protein binding**

Free (unbound) fractions of ARN-509 or MDV3100 in plasma were determined *in vitro* by equilibrium dialysis (BD Gentest, Woburn, MA) at 10 µg/mL in mouse and human plasma.

**GABA<sub>A</sub> binding**

GABA<sub>A</sub> binding experiments were carried out at CEREP, Paris, France. Briefly, membrane homogenates of cerebral cortex (120 µg protein) were incubated for 120 min at 22°C with 3 nM [35S]TBPS in the absence or presence of increasing concentrations of ARN-509 or MDV3100 in a buffer containing 50 mM Na<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub> (pH 7.4) and 500 mM NaCl. Nonspecific binding was determined in the presence of 20 µM picrotoxinin. Following incubation, the samples were filtered rapidly under vacuum through glass fiber filters (GF/B, Packard) presoaked with 0.3% PEI and rinsed several times with ice-cold 50 mM Tris-HCl using a 96-sample cell harvester (Unifilter, Packard). The filters were dried then counted for radioactivity in a scintillation counter (Topcount, Packard) using a scintillation cocktail (Microscint 0, Packard). The results were expressed as a percent inhibition of the control radioligand specific binding. The standard reference compound was picrotoxinin, which was tested in each experiment at several concentrations to obtain a competition curve from which an IC<sub>50</sub> was calculated.

**References**


