Targeting AR Variant–Coactivator Interactions to Exploit Prostate Cancer Vulnerabilities

Fiorella Magani1,2, Stephanie O. Peacock1,2, Meghan A. Rice1,2, Maria J. Martinez2, Ann M. Greene2, Pablo S. Magani2, Rolando Lyles1,2, Jonathan R. Weitz2, and Kerry L. Burnstein2,3

Abstract

Castration-resistant prostate cancer (CRPC) progresses rapidly and is incurable. Constitutively active androgen receptor splice variants (AR-Vs) represent a well-established mechanism of therapeutic resistance and disease progression. These variants lack the AR ligand-binding domain and, as such, are not inhibited by androgen deprivation therapy (ADT), which is the standard systemic approach for advanced prostate cancer. Signaling by AR-Vs, including the clinically relevant AR-V7, is augmented by Vav3, an established AR coactivator in CRPC. Using mutational and biochemical studies, we demonstrated that the Vav3 Diffuse B-cell lymphoma homology (DH) domain interacted with the N-terminal region of AR-V7 (and full length AR). Expression of the Vav3 DH domain disrupted Vav3 interaction with and enhancement of AR-V7 activity. The Vav3 DH domain also disrupted AR-V7 interaction with other AR coactivators: Src1 and Vav2, which are overexpressed in PC. This Vav3 domain was used in proof-of-concept studies to evaluate the effects of disrupting the interaction between AR-V7 and its coactivators on CRPC cells. This disruption decreased CRPC cell proliferation and anchorage-independent growth, caused increased apoptosis, decreased migration, and resulted in the acquisition of morphological changes associated with a less aggressive phenotype. While disrupting the interaction between FL-AR and its coactivators decreased N-C terminal interaction, disrupting the interaction of AR-V7 with its coactivators decreased AR-V7 nuclear levels.

Implications: This study demonstrates the potential therapeutic utility of inhibiting constitutively active AR-V signaling by disrupting coactivator binding. Such an approach is significant, as AR-Vs are emerging as important drivers of CRPC that are particularly recalcitrant to current therapies.

Introduction

Prostate cancer is highly prevalent and a leading cause of cancer deaths in men in the United States (1). Advanced prostate cancer is treated systemically by androgen deprivation therapy (ADT), which has been the standard of care for nearly 80 years since the recognition that prostate cancer is dependent on androgen receptor (AR) signaling for survival and growth (2–4). Despite symptomatic benefits, the disease frequently recurs as castration-resistant prostate cancer (CRPC; reviewed in refs. 5–7). Because in the majority of cases CRPC continues to rely on AR signaling, newer pharmacologic agents with improved capacity to block AR (enzalutamide) and androgen synthesis (abiraterone acetate) are used (8, 9). Despite these newer generation drugs, CRPC remains incurable.

One major mechanism underlying CRPC progression is the expression of constitutively active AR variants (AR-V) that lack the AR ligand binding domain and are therefore not readily targeted by current approaches (refs. 10–13; reviewed in ref. 14). AR-Vs retain the potent transactivating N-terminal domain (NTD), which is unique in the nuclear receptor family because of the presence of dominant activation motifs (15–17). Multiple AR-Vs have been discovered, but AR-V7 (also known as AR3 or AR1/2/3/CE3) is the most well-studied because it is readily detectable in clinical specimens (refs. 18, 19, reviewed in ref. 20). AR-Vs confer castration resistance in vitro and in vivo, and their presence in prostate cancer tumors and circulating tumor cells denotes poor prognosis (10, 18, 21–23). A recent study by Antonarakis and colleagues (24), which was performed in 202 patients, underlines the clinical significance of AR-V7 in human prostate cancer samples by demonstrating a correlation between AR-V7 levels and therapeutic resistance to ADT.

AR-Vs bind as homodimers or as heterodimers with full-length (FL) AR to androgen response elements (ARE) in chromatin (25, 26). The extent to which AR-Vs regulate unique genes (compared to full length AR) to drive prostate cancer progression is under active investigation (27–30). Because AR-V activity is critical for CRPC cell survival and resistance to even the newest generation of AR-targeted therapies, these variants are attractive targets for CRPC treatment (31). However, because AR-Vs lack the AR LBD, designing specific, high-affinity drugs is a major challenge (31). An alternative
approach is to impede the activity of AR-Vs by inhibiting their interaction with coactivators, many of which are overexpressed in CRPC (32–35).

We have previously demonstrated that AR and AR-V7 signaling is greatly enhanced by the coactivator Vav3 (35–37), a Rho GTPase guanine nucleotide exchange factor (GEF; ref. 38). Much like levels of AR-Vs, levels of Vav3 mRNA increase during progression to castration resistance in prostate cancer cell models, xenografts, and the Nkx3.1; Pten mouse prostate cancer model (32, 35, 39–41). Importantly, Vav3 protein levels are elevated in metastatic CRPC human specimens and are prognostic for posttreatment disease recurrence (42). We have also shown that Vav3 confers castration resistance in vitro and in vivo (36, 37). Here, we identified the domains of Vav3 and AR-V7 that interact, generated a reagent to disrupt this interaction, and observed the biological impact resulting from this disruption.

Further, we found that a closely related protein to Vav3, Vav2, is also overexpressed in human prostate cancer and enhanced AR and AR-Vs activity. We found that Vav protein interaction with the AR N-terminal Tau 5 domain is paradigmatic for other N-terminal interacting coactivators and was critical for AR/AR-V activity as well as CRPC cell survival, proliferation, and migration. This study provides proof-of-concept that disrupting the interaction between AR-Vs and their coactivators is a promising therapeutic strategy for CRPC.

Materials and Methods

Cell culture and chemical reagents

The human prostate cancer cell lines LNCaP (ATCC Catalog No. CRL 1740; batch F-11701), CWR-22Rv1 (CRL-2505, batch 4484055), and PC-3 (ATCC, Catalog No. CRL 1435; batch F-11154) were obtained from ATCC. CWR-R1, LNAI, ALVA31, and C4-2B cells were generous gifts from Dr. Elizabeth M. Wilson (University of North Carolina, Chapel Hill, NC), Dr. Priyamvada Rai (University of Miami, Miami, FL), Drs. Stephen Loop and Richard Ostensen (Department of Veteran Affairs Medical Center, Tacoma, WA), and Dr. Conor Lynch ( Moffitt Cancer Center, Tampa, FL), respectively. LNCaP, 22Rv1, CWR-R1, PC3, and ALVA31 DH-FLAG or empty vector linked to FLAG (EV-FLAG) cells were pools derived following transduction with the corresponding construct and selection using 500 mg/mL of G418 (Sigma). 22Rv1 and LNAI shVav2 cells were obtained from cells transduced with a PLKO.1 shVav2 plasmid and selected in 2.5 μg/mL puromycin (Sigma). Cell culture media (RPMI1640 and DMEM) were obtained from Cellgro by Mediatech, Inc. FBS was obtained from Atlanta Biologicals, Inc. LNCaP, ALVA31, 22Rv1, CWR-R1, and PC3 cell lines were cultured in RPMI supplemented with 100 IU/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L L-glutamine (Life Technologies, Inc.), and 10% FBS or 2% charcoal-stripped serum (CSS). C4-2B cells were cultured in DMEM supplemented with 100 IU/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L L-glutamine (Life Technologies, Inc.), and 10% FBS or 2% CSS. R1881 (methyltrienolone) was purchased from PerkinElmer Life and Analytical Sciences and used at 1 nmol/L. All cell lines were authenticated on February 2016 using STR (Genetica), and tested for Mycoplasma contamination every 6 months using the Mycoplasma PCR Detection Kit (Sigma; MP0035-1KT). All cell lines used were negative for mycoplasma, bacteria, and fungi contamination.

Plasmids

The following DNA constructs were generously provided: pcDNA3.1 ARv567es (Dr. Scott Plymate, University of Washington, Washington, DC), the constructs for the mammalian two-hybrid assay: Gal4DBD-ARLBBD, VP16AD-ARTAD, and Gal4-TataLuc (Dr. Karen Knudsen, Thomas Jefferson University), the ARE luciferase (ARE-luc; Dr. Zafar Nawaz, University of Miami), the MMTV and GRE luciferase plasmids (Dr. Mona Nemir, University of Ottawa, Ottawa, Canada), and the P5HB AR-V7 wild-type and deletion mutants (Dr. Scott Dehm, University of Minnesota, Minneapolis, MN). The nucleotide sequence used to target Vav2 mRNA (CCGGCGAATGGAACTCGAG-GAATTCGTGAGAATTCGTTTATTTTGTAGCAGTG) was cloned into a PLK0.1 vector. Vav3ADH was created via site-directed mutagenesis with the following primers: F- TCAAGCCTAATGCGAGGAGATGCAGATGACGACGATAA, R- AAGAATTC-GGTGTCGATGTGCGAGCTGAGA, R- TGTCCCTGGATGCTGGTCACCAAGAAAAGTCTGITT-GTGTCAAAATCTCATTTTCTGGACATTTGGGCTGA. DH-FLAG was isolated from Vav3 with the following primers: F- AAGCG-GCCCGATGATTACAGTGTTTCTTGACATTTGGGCTGA, R- AAGAATTC-TATA-GATGGTTTTACCATTTTACCAAGTTTCTCCAGGAGG.

Reporter gene assays and transfections

A dual plasmid mouse mammary tumor virus (MMTV)-luciferase system was used, in which one plasmid encodes wild-type MMTV promoter whereas the control plasmid lacks androgen/glucocorticoid response elements (AGRE). Non-AR-driven transcriptional activity and transfection efficiency can be accounted for by utilizing the AGRE plasmid as a baseline control. All transfections were carried out using Lipofectamine (Invitrogen Life Technologies) according to the manufacturer’s instructions. For luciferase assays, cells were plated at a density of 3.0 × 10^5 cells in 35-mm dishes 16 to 20 hours before transfection. Immediately before transfection, media were replaced with unsupplemented DMEM. For PC3 cells, each well was transfected with 1.6 μg of MMTV or AGRE reporter plasmids, and a combination of 250 ng pCMV-AR, pcDNA3.1ARv567, or p5HB-AR-V7; and 500 ng of pIRES-egfp-Vav2, pIRES-egfp-Vav3 wild-type, pIRES-egfp-Vav3 mutants, pIRES-SRC-1, or empty vector. DH interference was conducted in PC3, LNCaP, and C4-2B cells with 100 ng of pCMV-3tag1b-DH-FLAG or empty vector (pCMV-3tag1b-FLAG). For determining FL-AR N-C interaction (mammalian two-hybrid assay), PC3 cells were transfected with 500 ng of Gal4DBD-ARLBBD, VP16ADARTAD, and Gal4-TataLuc. DH interference was conducted in PC3, LNCaP, and C4-2B with 100 ng of pCMV-3tag1b-DH-FLAG or empty vector (pCMV-3tag1b-FLAG). After a 6-hour incubation with DNA/lipid complexes, cells were refed with RPMI supplemented with 2% (CSS) and treated with vehicle or 1 nmol/L R1881. Cells were harvested 48 hours after transfection, lysed, and assayed for luciferase activity using the Promega Luciferase Assay Kit (Promega Corp.). Luciferase assays in CWR-R1, LNCaP, C4-2B, and 22Rv1 were performed with the addition of a 5-minute DNA incubation with PLIUS reagent (Invitrogen Life Technologies). The experiments performed using the dual reporter plasmid MMTV or AGRE were analyzed by normalizing the reads of luciferase activity of each well to the protein amounts, and the activities from cells expressing the MMTV plasmids to those from cells expressing the AGRE control plasmid.

Luciferase activities were normalized to the protein amounts, and the activities from cells expressing the MMTV plasmids were...
normalized to those from cells expressing the ΔGRE control plasmid.

**Cellular fractionation**

Cells were plated at 2 × 10^5 cells per 100 mm dish and grown in 5% CSS for 72 hours. Cellular fractionation was performed with the Nuclear and Cytoplasmic Extraction Reagents (NE-PER #78833) according to the manufacturer’s protocol (Thermo Scientific). Ten micrograms of each protein sample were subjected to Western blot analysis as described below.

**Immunoblotting**

Cellular proteins were extracted and separated in 10% to 12% SDS-PAGE gels, and Western blot analyses were performed as previously described (36). The antibodies used were: anti-AR (N-20; 1:1000; Santa Cruz Biotechnology, Inc.), anti-AR-V7 (1:500; Precision Antibody), anti-Histone (1:1000; Santa Cruz Biotechnology, Inc.), anti-SOD (1:1000; Santa Cruz Biotechnology, Inc.), anti-cleaved PARP (1:1000; Cell Signaling Technology), anti-Vav3 (1:1,000, Cell Signaling Technology), anti-Vav2 (Santa Cruz Biotechnology, Inc.), anti-actin (1:500; Santa Cruz Biotechnology, Inc.), or anti-FLAG (1:1,000, Sigma). Densitometry was performed using ImageJ software (43).

**Cell proliferation assay (trypan blue)**

Cells were plated at an initial density of 20,000 per well in 24-well dishes. After 5 days, cells were trypsinized and viable cells were counted by trypan blue exclusion using a hemocytometer.

**RNA isolation and reverse transcriptase quantitative RT-qPCR**

Total RNA was collected using Trizol according to the manufacturer’s protocol (Life Technologies), and isolated using Direct-zol RNA MiniPrep Plus (Zymo Research, Catalog No. R2072). Total RNA was reverse transcribed using a cDNA Reverse Transcription Kit (Applied Biosystems, Catalog No. 4368814) as per the manufacturer’s protocol. TaqMan probes from Applied Biosystems for FKBPs, UBE2C, and GAPDH were used.

**Cell proliferation assay and apoptosis assay (IncuCyte)**

For growth assays, cells were plated in 96-well plates at 5,000 or 7,500 cells/well and transfected with 2% (v/v) of nonperturbing nuclear-restricted green fluorescent label (IncuCyte NucLight Green BacMam 3.0, Essen Bioscience). For apoptosis assays, cells were plated in 96-well plates at 10,000 cells/well and transfected with 1% (v/v) apoptosis marker reagent, which is cleaved by activated caspase 3/7, releasing a green fluorescent label (IncuCyte Caspase-3/7 Apoptosis Assay Reagent). After 2 hours, cells were incubated in an Incucytes Zoom (Essen BioScience), acquiring phase and green fluorescent images at 10x magnification every 2 hours. Incucytes Zoom software was used to analyze and graph the results.

**Soft agar assays**

Soft agar assays were performed as previously described (3). ALVA31, CWR-R1, and 22Rv1 plates were incubated for 2, 3, or 4 weeks, respectively. Colonies were stained with 0.005% crystal violet and counted using the Bio-Rad Gelledc system.

**Immunoprecipitation**

HEK293 cells were plated at 3.5 × 10^6 cells/100 mm dish. Cells were transfected with 5 μg of POCXIP AR-V7 or AR and pires-eGFP-Vav3-myc or pires-eGFP-Vav3-DCP-myc utilizing a Calcium-Phosphate Transfection Kit according to the manufacturer’s instructions (Clontech Laboratories). For DH interference, 22Rv1 and CWR-R1 cells were plated at 3 × 10^6 cells/100 mm dish and transfected with 10 μg DH-FLAG or EV using Lipofectamine reagent and Plus reagent (Invitrogen, Life Technologies). After 48 hours, cells were lysed and immunoprecipitation was performed as previously described (36), using nonspecific mouse IgG (2 μg, Santa Cruz Biotechnology, Inc.), monoclonal mouse antimyc (2 μg, Invitrogen) or anti-FLAG (2 μg, Sigma), nonspecific rabbit IgG (2 μg, Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti AR-N20 (2 μg, Santa Cruz, Biotechnology, Inc.), rabbit polyclonal anti-Vav2 (2 μg, Santa Cruz Biotechnology, Inc.), or rabbit polyclonal anti-Vav3 (2 μg, Millipore).

**Immunofluorescence, imaging, and analysis**

C4-2B cells were plated on glass cover slips in 24-well plates at 20,000 cells/well, and transfected with DH-FLAH or FLAG empty vector. After 48 hours, cells were fixed in 4% paraformaldehyde for 1 hour, permeabilized in 0.2% Triton X-100 for 10 minutes, and then incubated with Alexa Fluor 594-Phalloidin conjugate (Molecular Probes, Life Technologies). The coverslips were mounted using SlowFade Gold antifade reagent containing DAPI (Molecular Probes, Life Technologies) and imaged using a fluorescent microscope. Images were analyzed and cell body and protrusion lengths were measured using ImageJ software (NIH, Bethesda, MD).

**Analysis of human samples datasets**

The GSE56701 dataset was analyzed using Galaxy (44, 45) and the pipeline was modeled using Tophat aligner and Cufflinks to analyze mapped transcripts. GSE29650, GSE3325, and GSE6099 datasets were analyzed using the GEO2R online tool from ncbi. NIH.gov. The TCGA dataset (provisional dataset for prostate adenocarcinoma) was analyzed using cbioportal.org to build the Kaplan–Meier curves.

**Migration assays**

22Rv1 and C4-2B cells were serum-starved overnight and seeded at 20,000 cells/well in the top chamber of Boyden Chambers (8 μm pore size, BD Biosciences), and placed in 24-well plates. Media supplemented with 10% FBS was placed in the lower chambers as a chemoattractant. After 18 hours, cotton wool was used to remove non-migratory cells from the top chambers. Cells on the lower surface of the membrane were fixed in ice-cold methanol for 20 minutes, then stained with 0.01% crystal violet, and counted using a light microscope.

**Statistical analysis**

Data were graphed and analyzed using Prism 7 (GraphPad) and Statistica 8.0 (Statsoft). Data were tested for normality (Shapiro–Wilk test) and homogeneity of variances (Levene test). When both assumptions were met, data were tested for significance (P < 0.05) using a two-tailed Student T-test (two groups) or ANOVA (three or more groups). Otherwise, Welch’s correction or nonparametric statistical analyses were used: Mann–Whitney test (two groups) and Kruskal–Wallis (three or more groups).
Results

We previously showed that Vav3 increases the transcriptional activity of AR splice variants, including AR-V7 (36). To determine if endogenously expressed Vav3 interacted with AR-V7, we performed co-immunoprecipitations in the human CRPC cell line 22Rv1, which expresses both AR-V7 and Vav3. We observed that endogenous Vav3 and AR-V7 were present in the same immunocomplexes, and also found that Vav3 was co-immunoprecipitated with FL AR (Fig. 1A).

We next sought to identify the minimal necessary and sufficient regions of Vav3 required to enhance AR-V7 activity. We performed reporter gene assays using PC3 cells, a human AR-null prostate cancer cell line, transfected with an ARE-Luciferase reporter plasmid plus cDNAs encoding either FL-AR or AR-V7 and Vav3 truncation mutants. The N-terminus, C-terminus, or both of Vav3 were deleted to produce CaVav3, Vav3Dterm, and Vav3DPC, respectively (Fig. 1B). As expected based on our previous work, these three Vav3 mutants all retained the capacity for androgen-inducible co-activation of FL-AR (35, 37, 41, 46), but additionally, we found that these three Vav3 truncation mutants enhanced the ligand-independent activity of AR-V7 (Fig. 1C–E). Thus, the Vav3 DPC truncation mutant consisting of the DH, PH, and CRD regions retained the capacity to enhance AR-V7. Furthermore, Vav3-DPC interacted with both FL-AR and AR-V7 in co-immunoprecipitation experiments (Fig. 1F).

To refine further the Vav3 functional domains needed to enhance AR-V7 transcriptional activity, we generated two additional Vav3 mutants: Vav3ΔCRD, which lacks the cysteine-rich domain (CRD); and Vav3ΔDH, which lacks the DH domain, where the GEF catalytic activity resides (Fig. 2A). We found that Vav3ΔCRD retained the capacity to enhance the activities of both FL-AR and AR-V7 to the same extent as wild-type Vav3 (Fig. 2B). However, Vav3ΔDH was ineffective at enhancing either FL-AR or AR-V7 transcriptional activity compared to wild-type Vav3 (Fig. 2C), whereas there were no significant differences between Vav3 and Vav3ΔDH expression levels (Supplementary Fig. S1A). These data indicate that although Vav3-mediated co-activation of FL-AR and enhancement of AR-V7 is GEF-independent (35, 36), an intact GEF domain (DH domain) was needed.

Conversely, we mapped the region in AR that interacted with Vav3. Because AR-Vs lack the ligand-binding domain (LBD) and the AR N-terminal domain (NTD) is known to possess strong activation functions, we focused on the AR NTD. Within the NTD, the activation function-1 (AF-1) region is essential for AR transactivation and for interaction with several co-regulators (reviewed in refs. 47, 48). We examined the possibility that Vav3 would interact with the AF-1 region, which contains Transactivation Unit 1 (TAU1) and Transactivation Unit 5.
PC3 cells (AR-negative) were transfected with FL-AR or AR-V7, the reporter plasmid ARE-Luc, and a Vav3 mutant lacking the CRD (Vav3

Because Vav3 required its DH domain to interact with FL-AR and AR-V7, we examined if the DH domain of Vav3 was sufficient for this interaction. By performing co-immunoprecipitations, we determined that FL-AR and Vav3 DH domain linked to FLAG (DH-FLAG), and AR-V7 and DH-FLAG, were present in the same protein complexes (Fig. 3A and B). Once we established that both FL-AR and AR-V7 interacted with the Vav3 DH domain, we postulated that overexpressing this domain would interfere with FL-AR:Vav3 and AR-V7:Vav3 interactions and that disrupting these interactions would reduce Vav3-mediated enhancement of FL-AR and AR-V7 transcriptional activities. We found that expressing the Vav3 DH domain disrupted FL-AR:Vav3 and AR-V7:Vav3 interactions, as shown by co-immunoprecipitations (Fig. 3C). As expected, disruption of these interactions greatly reduced Vav3-mediated augmentation of FL-AR and blocked AR-V7 transcriptional activities (Fig. 3D). Expression of Vav3 DH domain did not affect endogenous Vav3 levels (Fig. 3E). These data show that expression of the Vav3 DH domain was sufficient to disrupt FL-AR:Vav3 and AR-V7:Vav3 physical interactions and enhancement of AR (FL and variant) transcriptional activities.

To extend our work on the role of Vav family proteins on AR signaling in PC, we sought to determine the role of Vav2 (Fig. 4A), which is closely related to Vav3 in terms of primary sequence and structure (49). To assess the possible relevance of Vav2 with respect to AR-V7 signaling in PC, we evaluated whether these genes were coexpressed in prostate cancer patient samples by querying two existing independent human datasets. The first dataset, from Hornberg and colleagues, 2011 (GSE29650; ref. 50), contained microarray data of 10 bone metastases samples from different prostate cancer patients with relatively high levels of AR-V7. Computational analysis revealed that Vav2, as well as Vav3 mRNAs, were present in all the AR-V7-high samples at significant, detectable levels (Fig. 4B). The coexpression of Vav2, Vav3, and AR-V7 mRNAs was confirmed for prostate cancer patient samples from Antonarakis and colleagues (GSE56701; ref. 51) in AR-V7-expressing circulating tumor cells (data not shown). The Vav2 gene is amplified in 10% of patient samples [Prostate Adenocarcinoma (Broad/Cornell, Nat Genet 2012, and TCGA dataset prostate adenocarcinoma)] and is overexpressed in 34% of prostate cancer patients samples in [Prostate Adenocarcinoma (MSKCC, Cancer Cell 2010)]. Moreover, analysis of the Varambally and colleagues 2005 dataset (GSE3325; ref. 52) revealed that Vav2 mRNA expression levels were elevated in primary prostate cancer patient samples compared to levels in normal prostate samples, and Vav2 mRNA levels were further elevated in metastatic prostate cancer samples (Fig. 4C). Similar results were obtained from the Tomlins and colleagues, 2007 dataset (GSE6099; ref. 53; data not shown). Interestingly, patients who presented higher levels of Vav2 at the time of prostate biopsy also exhibited decreased disease-free survival (DFS, P-value = 0.001; Fig. 4D), and reduced overall survival (data not shown, P-value = 0.0113; TCGA dataset prostate adenocarcinoma, from Chiportal.org).
Because of the potential significance of Vav2 in prostate cancer, we examined the effect of depleting Vav2 on 22Rv1 cell number and FL-AR/AR-V7 activity. Stable depletion of Vav2 using shRNA (Fig. 4E) reduced cell number (Fig. 4F) and decreased AR ligand-dependent and ligand-independent transcriptional activity (Fig. 4G). A similar result for ligand-dependent AR activity was observed in the CRPC cell line LNAI, a derivative of androgen-dependent LNCaP cells (data not shown). Co-immunoprecipitations in 22Rv1 cells revealed that endogenous Vav2 was co-immunoprecipitated with endogenous FL-AR and AR-V7 (Fig. 4H). Similar results were obtained in an additional CRPC cell line, CWR-R1 (data not shown).

As was observed for Vav3, we found that expressing the Vav3 DH domain disrupted FL-AR and AR-V7 transcriptional activities (Fig. 5A). We examined the effects of the Vav3 DH domain against a distinct and well-characterized AR coactivator, SRC-1 which is known to modulate AR and AR-V7 activities (54–56). Expression of the Vav3 DH domain blocked AR transcriptional enhancement by SRC-1 (Fig. 5B). Expression of only the Vav3 DH domain caused decreased FL-AR transcriptional activity (Fig. 5C) in LNCaP, a human cell line that contains high levels of the coactivator SRC-1 (54).
expression of the Vav3 DH domain reduced FL-AR N/C interaction (Fig. 5D).

Because the splice variant AR-V7 lacks the C-terminal LBD and is constitutively active, AR-V7 activity relies on AR-V7 presence in the nucleus, where it can enhance the transcription of downstream targets. We previously found that Vav3 increases nuclear levels of AR-V7 (36). 22Rv1 cells stably expressing DH-FLAG decreased androgen-inducible AR target gene (FKBP5) expression as well as ligand-independent expression of the AR-V7 target gene UBE2C (Fig. 5F).

Coactivators enhance FL-AR and AR-V7 transcriptional activity, while promoting an oncogenic transcriptional program that is thought to drive PC progression and metastasis (56, 58–61).

Therefore, we used the Vav3 DH domain for proof-of-concept experiments to study the cellular effects of disrupting the interaction of FL-AR and AR-V7 with endogenous coactivators that interact with both the AR and AR-Vs N-terminal domain. We generated LNCaP, 22Rv1, CWR-R1, PC3 (AR-null), and ALVA 31 (AR-null) cell lines stably expressing Vav3 DH-FLAG or its EV-FLAG. DH-FLAG was expressed at similar levels that interact with both the AR and AR-Vs N-terminal domain. Cell proliferation was measured by two techniques: in real-time using a live-cell imaging microscope (Incucyte Zoom, Essen Biosciences) and by trypan blue exclusion using a hemocytometer. Although LNCaP, 22Rv1, and CWR-R1 expressing Vav3 DH-FLAG exhibited decreased proliferation (Fig. 6A and Supplementary Fig. S1C), PC3 (Fig. 6A and Supplementary Fig. S1C), and ALVA31 (Supplementary Fig. S1C) cell viability was not affected, suggesting that the effects of the Vav3 DH domain were specific for AR. Expression of DH-FLAG did not decrease FL-AR (Supplementary Fig. S1E), AR-V7 (Supplementary Fig. S1E and S1F), Vav3 (Supplementary

Figure 4.

Expression of Vav2, a member of the Vav family, is elevated in human CRPC samples, and Vav2 interacts endogenously with and promotes the transcriptional activities of full length FL-AR and AR-V7. A, A schematic of Vav2 and Vav3 structure with the amino acid position of each domain. CH, calponin homology domain; AD, acidic domain; DH, diffuse B-cell lymphoma homology (GEF) domain; PH, Pleckstrin homology domain; CRD, cysteine rich domain; SH2, Src homology 2 domain; SH3, Src homology 3 domain. B, Vav3 and Vav2 mRNA levels are coexpressed in human CRPC bone metastases, which contain relatively high AR-V7 levels [dataset of Hornberg and colleagues (50)]. C, Vav2 mRNA levels are elevated in prostate cancer and metastatic prostate cancer compared to benign tissue [dataset of Varambally and colleagues (52)]. The upper curve denotes cases with no abnormal expression of Vav2, whereas the lower curve represents the cases in which Vav2 mRNA levels are upregulated (z-score threshold ± 2.0). $P$-value = 0.001. E, Immunoblotting was performed on 22Rv1 cell lysate using an anti-VAV2 antibody and anti-actin as the loading control. Data shown represent one of two independent experiments. F, Stable Vav2 depletion in 22Rv1 cells (vs. control shGFP) decreased cell number. Data shown represent two independent experiments performed in triplicate. Independent Student T-test, $P$-value < 0.001. G, 22Rv1 cells were transfected with the dual plasmid luciferase reporter system: MMTV or ΔGRE described in Materials and Methods. Luciferase activity was determined 48 hours after transfection. Data shown represent three independent experiments performed in triplicate, showing the mean ± SE, and normalized to their shGFP controls. Unpaired T-test, $P$-value = 0.001 in the presence of androgen; and $P$-value < 0.001 in the absence of androgen. H, 22Rv1 cells were harvested and co-immunoprecipitations were performed using antibodies to rabbit IgG control or Vav2. Immunocomplexes were immunoblotted with antibodies against FL-AR or AR-V7 (*, $P$-value < 0.01; **, $P$-value < 0.001).
Fig. S1F), or Vav2 levels (Supplementary Fig. S1G). We found that disrupting the interaction between AR and its coactivators rendered cells more rounded and with shorter total protrusions for each cell, normalized to cell body length.

To determine the role of the interaction between AR and coactivators in cell aggressiveness, we examined cell migration and morphology. Using Boyden chamber assays, we found that disrupting the interaction between FL-AR and AR-V7 with their coactivators decreases anchorage-dependent and -independent growth, and increases apoptosis.

To determine the role of the interaction between AR and coactivators in cell aggressiveness, we examined cell migration and morphology. Using Boyden chamber assays, we found that disrupting the interaction between FL-AR and AR-V7 with their coactivators reduced cell motility in 22Rv1 (Fig. 7A) and C4-2B (data not shown) cells, and changed cell morphology. Using fluorescent phalloidin staining for F-actin, data were quantified from four independent experiments plotted as the mean ± SE (Student’s t-test, P-value < 0.05).

The expression of FKBPS and UBE2C was examined in 22Rv1 cells transfected with DH-FLAG or EV by RT-qPCR analysis and normalized to GAPDH mRNA. Hormonal induction was the ratio of FKBPS mRNA levels from cells stimulated with R1881 compared to vehicle control treated cells (unpaired t-test, P-value = 0.03). For UBE2C quantification, cells described above were cultured in 2% CSS (unpaired t-test, P-value < 0.01). A representative experiment of two independent experiments performed in triplicate is shown (**, P-value < 0.01; *, P-value < 0.05).
protrusions (Fig. 7C), consistent with less motile cells and a less aggressive phenotype.

**Discussion**

As expression and function of AR-V7 (and other constitutively active AR variants) is a key mechanism underlying resistance to current PC treatments (10, 18, 21–24), it is imperative to develop novel AR inhibitors that target the N-terminal domain, which is present in both FL-AR and AR splice variants (reviewed in 51). Because AR-Vs may function either as homodimers or heterodimers with FL-AR (13, 22) and promote ligand-independent AR activity (13, 25, 26), therapeutic modalities must address both possible modes of AR-V action. Targeting the AR DNA-binding domain (DBD), common to FL and AR-Vs, has been pursued. Given that the DBD is the most conserved domain across the entire steroid/nuclear receptor family, generating selective inhibitors is challenging. The AF-1 region, located in the N-terminal domain, is also shared by FL-AR and AR-Vs and is critical for AR transcriptional activity (48). Efforts to target this region are also underway. However, the NTD has a disordered structure making it difficult to design selective inhibitors. Our results support the importance of channeling research efforts towards targeting the AR AF-1 region, because this would disrupt AR interaction with coactivators, in turn preventing FL-AR N-C interaction and decreasing AR-V7 nuclear levels, leading to decreased cell proliferation and acquisition of an aggressive phenotype.

Here, we show for the first time that Vav3 interacted via its DH domain with endogenous FL-AR and AR-V7 to enhance AR transcriptional activity. The Vav3 binding site on AR was mapped to the TAU5 region in the AR AF-1 domain, although additional interaction sites are possible. We also identified a novel AR coactivator: Vav2, another member of the Vav family, which is upregulated in PC human samples, and is prognostic for poor outcome. Like Vav3, Vav2 enhanced and interacted with endogenous FL-AR and AR-V7. We used the expression of the Vav3 DH domain, which consists of 178 amino acids and adopts a distinct structure, in a proof-of-principle approach to evaluate the effects of disrupting FL-AR and AR-V7 interaction with ectopic or endogenously expressed coactivators in multiple prostate cancer cell lines. Disruption of these critical interactions decreased AR-expressing prostate cancer anchor-age-dependent and anchorage-independent proliferation, increased apoptosis and decreased migration accompanied by morphological changes. Most importantly, these effects were specific to prostate cancer cells expressing AR, because expression of the Vav3 DH domain had no effect in two distinct AR-null PC cell lines. These results are in agreement with those reported by Nakka and colleagues (56); who demonstrated that disrupting the FL-AR and the p160 coactivator interface is a sound therapeutic approach in CRPC. Our results extend these findings by demonstrating the importance of targeting AR N-terminal-interacting coactivators to reduce AR and AR-V7 transcriptional activity, PC cell growth, survival,
Mechanistically, we found that FL-AR interaction with coactivators depended on the well-characterized interaction between the AR amino and carboxyl termini N-C interaction (critical for its activation and transcriptional activity). However, disruption of AR-V7 interaction with coactivators led to decreased AR-V7 nuclear levels. Because disrupting AR-V7 interaction with the TAU5 region of AR-V7 may increase AR-V7 stability in the nucleus. This increased stability may be achieved by protecting AR-V7 ubiquitination sites from detection, delaying AR-V7 degradation in the nucleus, and thus prolonging its transcriptional activity. In addition, AR-V7 interaction with coactivators such as Vav2 and Vav3 may also disrupt and compete with inhibitory factors, such as FOXO1, for the AR-V7 TAU5 binding site (62).

Peptidomimetic conjugates (MCPs) are peptoids that display bioactive ligands and are resistant to proteases (63, 64). Such conjugates have been used to antagonize AR. MCPs, such as MCP6, prevent intermolecular interactions between AR and its coactivators by changing the AR conformation (64). However, MCP6 shows no effect in the human CRPC cell line 22Rv1. The reason MCP6 fails to inhibit AR-V-containing 22Rv1 may be that MCP6 competes for androgen binding to the AR LBD (64), and may not interfere with binding of coactivators to the NTD. Similarly, the peptidomimetics used by Ravindranathan and colleagues (65) disrupt the interaction between FL-AR LBD and its coactivators. However, these peptidomimetics are unable to inhibit AR-V7 activity. Our results support the need for novel inhibitors against the AR NTD with the capacity to target both FL-AR as well as AR-Vs and that are effective even in the setting of overexpression of AR coactivators, as we have modeled here.

This study indicates that targeting the interaction between FL-AR or AR-V7 and their coactivators is sufficient to inhibit prostate cancer and CRPC proliferation, as well as survival and migration, and thereby could serve as a therapeutic modality in advanced disease.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: F. Magani, S.O. Peacock, K.L. Burnstein Development of methodology: F. Magani, S.O. Peacock, M.A. Rice, K.L. Burnstein Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): F. Magani, S.O. Peacock, M.A. Rice, A.M. Greene, R. Lyles, K.L. Burnstein Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): F. Magani, S.O. Peacock, M.J. Martinez, P.S. Magani, R. Lyles, J.R. Weitz, K.L. Burnstein
References


Grant Support

This work was supported by R01CA132200 (to K.L. Burnstein), Sylvester Comprehensive Cancer Center Developmental funds (to K.L. Burnstein), and F30AG038275 (to S.O. Peacock). Research performed in this manuscript was supported by NIH grant CA132200 (to K.L. Burnstein), NIH pre-doctoral fellowship F30AG038275 (to S.O. Peacock), and developmental funds from the Sylvester Comprehensive Cancer Center.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 23, 2017; revised July 21, 2017; accepted August 10, 2017; published OnlineFirst August 15, 2017.


Targeting AR Variant–Coactivator Interactions to Exploit Prostate Cancer Vulnerabilities

Fiorella Magani, Stephanie O. Peacock, Meghan A. Rice, et al.


Updated version Access the most recent version of this article at: doi:10.1158/1541-7786.MCR-17-0280

Supplementary Material Access the most recent supplemental material at: http://mcr.aacrjournals.org/content/suppl/2017/08/15/1541-7786.MCR-17-0280.DC1

Cited articles This article cites 64 articles, 24 of which you can access for free at: http://mcr.aacrjournals.org/content/15/11/1469.full#ref-list-1

Citing articles This article has been cited by 1 HighWire-hosted articles. Access the articles at: http://mcr.aacrjournals.org/content/15/11/1469.full#related-urls

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link http://mcr.aacrjournals.org/content/15/11/1469. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.