Developing a Model System to Study the Mechanisms of Resveratrol Inhibition of AR-V7 Transcriptional Activity

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Developing a Model System to Study the Mechanisms of Resveratrol Inhibition of AR-V7 Transcriptional Activity

A thesis in Biomedical Biochemical Sciences by Sarah R. Wilson

Submitted in Partial Fulfillment of the Requirements for the Degree of Master’s in Biomedical Sciences

August 2016
Signatory Page

We have read, examined, and approved this thesis.

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Abstract

Prostate cancer is one of the biggest threats to men’s health in the western world and it accounts for the second largest number of male cancer-related deaths in the United States. It is well established that prostate cancer cells depend on the androgen/androgen receptor pathway. Therefore, androgen deprivation therapy (ADT) has become the primary treatment option for prostate cancer. Patients with metastatic prostate cancer who receive (ADT) have shown increased quality of life. However, survival benefit with ADT is reduced dramatically in castration-resistant prostate cancer (CRPC). Androgen receptor (AR) continues to be functional in CRPC through various mechanisms. It is becoming evident that AR-V7, an AR variant with constitutive transcriptional activity, plays crucial roles in the development of CRPC. In order to study the mechanism of AR-V7’s actions and the ability of resveratrol to repress AR-V7, we ectopically expressed AR-V7 in PC3 cells and treated the cells with different concentrations of resveratrol. Resveratrol represses AR-V7 transcriptional activity in a dose-dependent manner evidenced by levels of the AR target gene PSA. Mechanistically, we found that under our specific experimental conditions resveratrol down-regulates AR-V7 protein levels post-transcriptionally without affecting AR-V7 subcellular location. Given the fact that resveratrol potentially represses endogenous AR-V7 transcriptional activity at the transcriptional level, resveratrol could become an option in CPRC treatment.
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Introduction:

Approximately 220,800 new cases of prostate cancer are diagnosed in men in the United States every year, among them more than 27,000 were predicted to die from prostate cancer this year\(^1\). Therefore, not only is prostate cancer a common disease among men, it is also deadly. In addition, it is now clear that prostate cancer is a disease of aging\(^2\)\(^4\). Given the fact that the baby boomers (people born between the years 1945 and 1965) have entered the high prostate cancer incidence bracket\(^5\), it is expected that prostate cancer diagnoses will increase as a large portion of the United States population ages. Prostate specific antigen (PSA) has been widely used as a prostate cancer screening tool\(^6\). Prostate biopsy techniques have recently become more sensitive. If prostate cancer is caught in the early stages and treated timely, the 5-year survival rate is about 98\%\(^1\). Advances in these diagnostic procedures will undoubtedly lead to an increase in prostate cancer diagnoses. Unfortunately, almost all prostate cancers eventually progress into an aggressive and extremely lethal form of cancer known as castration resistant prostate cancer (CRPC). Currently, there are limited reliable treatment options for CRPC\(^7\). Thus, it is imperative to understand the underlying mechanisms in the development of CRPC and develop efficacious strategies in CRPC therapy.

Androgens, a family of cholesterol-derived steroid hormones, are found in both men and women. In women, testosterone is produced mainly in the ovaries and is a precursor of oestradiol\(^8\). In males, testosterone is mainly produced in the testes and adrenal gland and plays an important role in both prostate physiology and prostate
cancer development. Once testosterone enters the cells it is usually converted to a more potent form, 5alpha-dihydrotestosterone (DHT). Both testosterone and DHT then interact with and function through androgen receptors (AR). AR is a nuclear receptor composed of three functional domains, the amino-terminal trans-active domain, the central DNA-binding domain, and the carboxyl-terminal ligand-binding domain. The amino-terminal domain has numerous amino acid repeats and two separate regions that are mandatory for full transcriptional activity. The central DNA-binding domain is structured in a helix-loop-helix manner and interacts with unique DNA sequences, androgen response elements (AREs). The C-terminal ligand-binding domain binds the ligand (androgen). Androgens (such as DHT) bind to the ligand-binding domain of the AR which leads to the formation of androgen/AR complexes. The androgen/AR complexes then form homodimers and translocate to the nucleus. In the nucleus, AR homodimers bind to androgen response elements (AREs) in the promoter/enhancer regions of target genes resulting in specific transcriptional regulation of AR targets (reviewed by Feldman and Feldman) (Figure 1).

The primary treatment for early stage prostate cancer is to directly target androgen-regulated pathways via androgen deprivation therapy (ADT). Surgical orchiectomy is an androgen deprivation option, but the psychological impact of surgical castration deters many patients from this option. Non-surgical ADT either suppresses the production of androgens or disrupts androgen/AR interactions. ADT has shown to be effective in increasing quality of life in patients with metastatic prostate cancer; patients reported decreased urethral obstruction, spinal cord
compression, and pathological fractures. Unfortunately, ADT has not proven to increase long-term survival\textsuperscript{12}. Most prostate cancers will eventually develop into an aggressive form of prostate cancer that is termed castration resistant prostate cancer (CRPC). CRPC is less hormone-dependent, however, AR signaling remains functional\textsuperscript{13,14}. Currently, there is no cure for CRPC\textsuperscript{7}. A variety of changes to AR have been shown to contribute to its ability to function despite androgen deprivation, including AR gene amplification/overexpression, intra-tumoral androgen synthesis, over-expression of AR co-activators, aberrant kinase pathway activation, AR mutation, and expression of constitutively active AR splice variants\textsuperscript{15}. Mechanistically, AR splice variants have been described as the product of AR gene rearrangements, and alternative splicing\textsuperscript{16,17}. Among all the AR splice-variants identified so far, AR Variant-7 (AR-V7) is the most common and the best-studied variant in CRPC\textsuperscript{17-19}. AR-V7 and AR-FL transcripts are increased in CRPC with AR-V7 significantly more increased than AR-FL\textsuperscript{20}. AR-V7 is expressed in basal and stromal cells and very weakly in luminal epithelial cells of healthy human prostate tissue\textsuperscript{21}. AR-V7, along with the other 16 known variants, lacks the ligand-binding domain (LBD) but retains transcriptional activity in the absence of androgens. Whether the unique 16 amino acid sequence at the C-terminus of AR-V7 plays a role in its constitutive activity is unknown (reviewed by Ware et al.,)\textsuperscript{22}. The full-length AR (AR-FL) and some commonly identified AR variants are shown in Figure 2. Interestingly, AR-FL has a hinge region located between the ligand binding domain and the DNA binding domain that houses a nuclear localization signal. AR-V7 lacks a hinge region but remains translocated in the nucleus. In addition, AR-V7 lacks a nuclear export
signal encoded in the LBD of AR-FL, this could also be involved in AR-V7 nuclear location\textsuperscript{23}. In overexpression experiments \textit{in vitro} AR-V7 homodimers and AR-V7/AR-FL heterodimers were found primarily localized within the nucleus. Further experimentation and \textit{in situ} experiments need to be done to determine the relationship and mechanism between AR-V7 and AR-FL nuclear localization\textsuperscript{24}. Recent studies have shown that not only do AR-variants activate transcription of AR-FL target genes, such as PSA\textsuperscript{25}, but they also have a unique set of transcription targets, such as AKT1\textsuperscript{21}.

Figure 1: Androgen-mediated AR molecular functions. Interaction between androgens and AR leads to AR dimerization and nuclear translocation. In the nucleus, AR binds to the AREs in target gene promoter/enhancer regions resulting in specific regulation of the AR target gene expression. In prostate cancer cells AR increases cellular proliferation, survival and metastasis. PSA is a well-established AR target\textsuperscript{10}, “Reproduced with permission from The New England Journal of Medicine, Copyright Massachusetts Medical Society.” (Nelson: Targeting the Androgen Receptor in Prostate Cancer — A Resilient Foe)
Secondary and tertiary prostate cancer therapies are used to slow the progression of CRPC. Mechanistically, the secondary prostate cancer therapies work in two ways. They either prevent the synthesis of extra-gonadal androgens (abiraterone) or inhibit AR signaling (enzalutamide). Abiraterone functions by inhibiting CYP17, one of the key enzymes in androgen synthesis, it functions in the adrenal glands, testes, and the prostate tumor. Enzalutamide competes with androgens by binding to the ligand binding domain of AR. Enzalutamide disrupts the interaction between androgen and AR thus impairing AR nuclear translocation as well as AR transcriptional activity. Taxane-chemotherapy is the third line of prostate cancer therapy utilized in the relatively early stages of CRPC. Taxane-chemotherapy prevents AR from translocating to the nucleus. Increased AR-V7 expression has been correlated with cancer resistance to enzalutamide and abiraterone. It was
recently discovered that when AR-V7 is present, taxane-therapy is unable to prevent AR-FL nuclear translocation\textsuperscript{31}. These lines of evidence suggest that targeting AR-V7 could be an efficacious strategy in designing a fourth class of drugs against AR-V7-positive CRPC.

Resveratrol (RSV), one of the phytoalexins found in numerous food sources including peanuts, grapes, and red wine has been shown to interfere with cellular events associated with cancer initiation, promotion, and progression\textsuperscript{32,33}. SIRT1 is a NAD$^+$-dependent histone deacetylase that functions by removing the acetyl group of lysine residues in histone tails rendering a more condensed chromatin formation that hinders transcription\textsuperscript{34}. Interestingly, RSV appears to bind to SIRT1, inducing a structural change allowing enhanced binding of substrate to lower the Km, and enhanced deacetylation activity of SIRT\textsuperscript{35}, thus further hindering transcription. Additionally, PTEN tumor suppressor is a target of select oncomiRs in prostate cancer cells. RSV appears to restore PTEN tumor suppressor capability by inhibiting select oncomiRs\textsuperscript{36}. Results from previous research have demonstrated that RSV can down-regulate the expression of both AR-FL and AR target genes including PSA\textsuperscript{37}. RSV has also been shown to reduce the level of AR-FL protein post-translationally\textsuperscript{33}. The mechanism behind RSV effects on AR-FL is not completely known. However, based on the similarity between AR-FL and AR-V7, we hypothesize that RSV may also affect AR-V7 transcriptional activity. In this research, we expressed AR-V7 in PC3 cells, a well-studied prostate cancer cell line that lacks androgen receptors and is thus androgen-independent\textsuperscript{38}, and tested the effects of RSV on AR-V7
transcriptional activity by measuring the mRNA levels of AR target gene PSA. Mechanistically, we found that RSV inhibits AR-V7 transcriptional activity by down-regulating AR-V7 protein levels post-transcriptionally.
Materials and Methods

Reagents

Ham’s F-12 (1x) with L-glutamine, RPMI (1x) with L-glutamine, and Opti-MEM (1x) with HEPES, 2.4g/L sodium bicarbonate, and L-glutamine culture media were purchased from Mediatech, Inc. (Manassas, VA). Antibiotic-Antimycotic (100x) was purchased from Life Technologies. Both media and Antibiotic-Antimycotic (100x) were stored at 4°C. Premium Fetal Bovine Serum (FBS) was purchased from Atlanta Biological (Oakwood, GA) and was stored at -20°C. Trypsin was purchased from Mediatech, Inc and stored at -20°C (0.25%, 2.21mM EDTA). PC3 and LNCaP cells were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). RSV was purchased from Sigma-Aldrich and was stored in the dark at -30°C. RSV was dissolved in 100% ethanol (Biotechnology Grade Anhydrous-Alcohol, IBI Scientific, Peosta, IA) to make 50mM, and 200mM stock solutions. The PCR and qPCR primer pairs for PSA, GAPDH, and AR-V7 were purchased from Invitrogen (Carlsbad, CA) and stored in the dark at -30°C. Primer pairs were diluted with reagent-grade double distilled (RGDD) water to make 100μM stock solutions. AR Antibody (N-20) (Santa Cruz Biotechnology Dallas, TX) was stored at 4°C. RIPA buffer was stored at 4°C and was prepared with 1% (v/v) NP40 (Novex by Life Technologies), 150 mM NaCl, 50mM Tris-Cl, 0.50% DOC, 1mM PMSF, 0.1% SDS, and 1mM DTT (Sigma-Aldrich). Lysogeny Broth (LB broth) was prepared with 10g bactotryptone, 5g bacto-yeast extract, and 10g NaCl/liter. LB broth was stored at 4°C. Geneticin (G4-18 sulfate) was purchased from Gold-Bio and stored in the dark at 4°C. G4-18 sulfate was mixed with Ham’s F-12 media to make a concentration of
100μM/mL and stored at 4°C. Ampicillin was purchased from Sigma-Aldrich and stored at 4°C. Ampicillin was mixed with RGDD to make a 25mg/mL stock solution and was stored at 4°C. RE-core 10X buffer, restriction enzymes ECoR1, Kpn1 and Xho1 were purchased from Promega and stored at -30°C.

Cell Cultures
PC3 cells were cultured in Ham's F-12 with L-glutamine, 10% premium FBS, and 1% Antibiotic-Antimycotic. LNCaP cells were cultured in RPMI 1640 with L-glutamine, 10% Premium FBS, and 1% Antibiotic-Antimycotic. Cells were kept in an incubator at 5% CO₂ and 37°C.

Plasmid preparation
The pcDNA3.1-AR-V7 plasmid, a gift from Dr. Luo's laboratory (John's Hopkins University), was on a filter paper. The filter paper was soaked in 30μL of RGDD overnight. Fifty microliters of chemically competent DH5α-E. coli cells (Invitrogen) were thawed from -80°C. PC3.1-AR-V7 cDNA was transformed into the chemically competent DH5α-E. coli cells. Briefly, plasmid was mixed with the bacterial cells, incubated on ice for 30 minutes, and then incubated at 42°C for 1.5 minutes. Two hundred microliters of LB broth was added and the mixture was placed in a shaker for one hour at 30°C. Approximately 200μL of transformants were plated on an ampicillin-containing agar plate and incubated overnight at 37°C. A colony was inoculated in LB broth with 100μg/ml ampicillin and cultured overnight. The bacterial cells were collected by centrifugation at 4°C, 2500 RPM for five minutes
and the plasmid was isolated using a QIAprep Spin Miniprep Kit purchased from Qiagen (Valencia, CA). Plasmid DNA was quantified using Nanodrop 2000 and the quality was assessed by mixing 7μL of plasmid with 2.5μL 4x loading buffer and loaded on a 1-2% agarose gel.

**Transfection**

PC3 cells with approximately 90% confluent on 10cm plates were suspended in Ham’s F-12 media and distributed evenly onto 10cm plates or into a six-well plate (Chemglass Life Sciences, Vineland, NJ) and cultured for 24 hours creating approximately 80% confluent plates. The cells were washed with 1mL or 6mL of Opti-MEM media for 6-well or 10cm plate, respectively. Lipofectamine 2000 and plasmid were diluted in the appropriate amount of Opti-MEM media per manufacturer protocol and incubated for 5 minutes after gently mixing, 4μg and 12μg of AR-V7 plasmid was used for six-well and 10 cm plates, respectively. The mixture was incubated at room temperature for twenty minutes. The lipofectamine-plasmid DNA complex was added dropwise to the appropriate well or plate. After 4-6 hours of incubation, growth media was added with or without RSV and further cultured for 24-48 hours. Cells were collected and the cell pellets were stored at -30°C until use.

**Cell line establishment**

Restriction enzyme 10x buffer, reagent grade double distilled water, 5μg of 0.3494μg/μl DNA, and EcoR1 or, Kpn or, Xho1 restriction enzyme were mixed and
incubated at 37°C for three hours in order to linearize the pcDNA3.1 AR-V7-expressing plasmid. Linearized DNAs were separated on 2% agarose gel. To precipitate the DNA, RGDD was added to the linearized DNA to make a 200µL volume. Twenty microliters of 3M NaOAc was added and mixed by inverting. Four hundred microliters of 100% EtOH was added and vortexed for ten seconds to mix. The mixture was incubated at -70°C for 20 minutes then centrifuged for 20 minutes at 4°C, 15000 RPM. Supernatant was aspirated and the pellet was washed with 500µL 70% EtOH. The pellet was left at room temperature to dry then re-suspended in 10µL of RGDD. Linearized AR-V7-expressing plasmid (0.0862 µg/µl) was transfected into PC3 cells using 4.0µL of lipofectamine. One hundred micrograms per milliliter of Geneticin in Ham’s media was added every 2-3 days for 2.5 weeks to select for AR-V7 expressing cells.

**Total RNA purification and Reverse Transcription-PCR (RT-PCR)**

RNA was purified from cells using RNeasy Plus Micro Kit (Qiagen Venlo, Limburg, Netherlands) per manufacturer’s protocol. RNA concentrations were estimated using NanoDrop 2000c UV-Vis Spectrophotometer, 5µg of RNA were then reverse transcribed using a Reverse Transcriptase Kit (SuperScript II (Invitrogen)), per manufacturer’s protocol. Resulting cDNA was diluted in RGDD and stored at -30°C. Polymerase Chain Reaction (PCR) was conducted using the Promega GoTaq Flexi DNA Polymerase kit (Madison WI) with primers specific for PSA, AR-V7, and GAPDH using 1µg of cDNA. The Techne thermal-cycler (Staffordshire, UK) was used for PCR reactions: denatured for two minutes at 95°C, 29-34 cycles of denature (95°C, 1
minute), annealing/extension (60°C, 1 minute) and extension/elongation (72°C, 1 minute). A 1% agarose gel was made using ultrapure agarose (Invitrogen) and 1x TAE buffer. All-purpose Hi-Lo DNA Marker (Bionexus, Oakland CA) was used as a ladder. Samples were loaded into gel wells and ran for 30-45 minutes at 59-82 volts and visualized under the UV-light.

<table>
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<th>Primer Type</th>
<th>Target</th>
<th>Forward</th>
<th>Reverse</th>
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<td>AGCACCTGCCAGGGTTGGGA</td>
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<td>PCR AR-V7</td>
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<td>TTTGAATGAGGCAAGTCAGCCTTCT</td>
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<tr>
<td>PCR GAPDH</td>
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<td>ACCACTGACACGTGGGCAGT</td>
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<tr>
<td>PCR ANKH</td>
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<td>CCACTGATGTCTATCAA</td>
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<td>AAATGAGCCCAAGTCTTCTC</td>
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</tr>
</tbody>
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Table 1: Primers for RT-PCR or qPCR.

**Quantitative PCR (qPCR)**

The qPCR reactions were prepared based on the protocol and reagents of the SYBR Advantage qPCR Premix (Clontech Laboratories, Inc., Mountain View, CA) in triplet-quintet in 96-well qPCR plate (Applied Biosystems, Life Technologies). After brief centrifugation, the plate was loaded into the ABI PRISM 7000 Sequence Detection System. GAPDH was labeled as an endogenous control and PSA was labeled as a target. The PCR was conducted as: hot start (1 cycles at 95°C for 30sec), 40 cycles of (denature at 95°C for 5sec and extension at 60°C for 37sec).
Preparation of whole cell lysate, nuclear and cytoplasmic extracts, and western blot

Lysing the cells in RIPA buffer and centrifuging for 30 minutes at 15000 RPM for whole cell lysates. To prepare nuclear and cytoplasmic extracts, cells were collected and Life Technologies nuclear extraction kit was used per manufacturer’s protocol. Protein concentrations were estimated using the Pierce BCA Protein Assay Kit (Thermo Fischer Scientific Waltham, MA). Using the iBlot system (Invitrogen) 15-90µg of total proteins was separated on polyacrylamide gels (4-5% stacking, 10% separating). Then the proteins were transferred to iBlot Nitrocellulose transfer stacks (Novex by Life Technologies), ponceau red staining (Sigma-Aldrich) verified transferred proteins. Blots were blocked in Fast Western Antibody Diluent (Thermo Fisher Scientific) for 1-2 hours and incubated overnight with primary antibodies at 4°C. Blots were washed in PBS-Tween 3x10 min and incubated with secondary antibody conjugated with HRP and clarity western ECL substrate (Bio-Rad Laboratories, Hercules CA). Image J software was used to quantify the intensities of the protein bands.

Immunostaining

Coverslips were washed with 70% EtOH, dried, and coated with poly-D lysine for 20 minutes. The coverslips were placed in 10cm or 6-well plates and cells were seeded to create approximately 80% confluence and incubated overnight. Coverslips were rinsed with PBS+Ca+Mg, cells were fixed with 4% paraformaldehyde for 20 minutes
at room temperature. Cells were washed with PBS 3 times, 3 minutes each. Cells were then permeabilized and blocked with 250μL of 0.3% triton, and 2% goat serum in PBS for 30 minutes. Cells were incubated with primary antibody in 1.5% goat serum in PBS for one hour at room temperature, washed three times in PBS, 10 minutes each time. Cells were then incubated with red fluorophore conjugated secondary antibody in 1.5% goat serum for 30 minutes at room temperature in dark, and rinsed one time with PBS. Hoescht 33258 5μg/mL in PBS was used to stain nuclei for ten minutes in the dark at room temperature and washed 3 times in PBS, ten minutes each. Vectashield was added to a slide and the coverslip was placed facing down. Cells were stored at 4°C in the dark for analyses.
Results

**Ectopically expressed AR-V7 in PC3 cells is a functional transcriptional factor**

PC3 is a prostate cancer cell line that does not express endogenous AR. We decided to ectopically express AR-V7 in this cell line to establish a system for testing AR-V7 function in a prostate cancer model without the effects of AR-FL. For this purpose, PC3 cells were transiently transfected with a plasmid expressing AR-V7. Expression of PSA, a well-known transcriptional AR target gene was evaluated by RT-PCR to determine if the ectopically expressed AR-V7 was functional. The cells were collected 48 hours after transfection, total RNA was purified, and RT-PCR was conducted. As shown in Figure 3A, AR-V7 mRNA was detected in the transfected, but not the un-transfected, PC3 cells, indicating that the transfection worked as expected. Transfection of plasmid and expression of AR-V7 had no effect on the housekeeping gene GAPDH. However, the PSA mRNA level was up regulated only in the transfected PC3 cells. LNCaP is a prostate cancer cell line that expresses endogenous AR-FL with readily detectable levels of PSA mRNA. Therefore, RNA purified from LNCaP cells was used as positive control for RT-PCR and water was used as a negative control. As shown in Figure 3B both PSA and GAPDH were only observed in the positive, but not the negative control. Extremely low levels of AR-V7 have been reported in the LNCaP cells, but it was not detectable under the conditions used in our research.
Figure 3: Ectopic AR-V7 expression in PC3 Cells RT-PCR: (A) PC3 cells were transfected with (lane 1) and without (lane 2) AR-V7-expressing plasmid. After 48 hours, cells were collected, total RNA was purified, and RT-PCR was done to estimate AR-V7 (29 cycles) and PSA (34 cycles) mRNA levels. (B) Total RNAs from LNCaP cells were used in RT-PCR as positive control and RGDD serves as a negative control. GAPDH serves as an internal control.

In order to further confirm that the ectopically expressed AR-V7 was functional, we conducted qPCR with the same RNAs used in RT-PCR. Consistent with the results from the RT-PCR (Figure 3), the results from qPCR demonstrated that compared to the un-transfected control, the PSA mRNA is up-regulated by overexpressed AR-V7 (Figure 4). One experiment was performed in quintet; further experimentation needs to be done to establish statistical significance. This data collectively demonstrated that the ectopically expressed AR-V7 is functional in up-regulating the AR target gene PSA. This suggests that this AR-V7 system can be used to test different chemical’s effects on AR-V7 transcriptional activity.
Figure 4: Ectopic AR-V7 expression in PC3 Cells qPCR: PC3 cells were transfected with and without AR-V7-expressing plasmid. 48 hours after transfection, cells were collected, total RNA was purified, and qPCR measuring raw relative quantification (RQ) was conducted. A delta-delta CT study was performed using GAPDH as the internal control and PSA as the target gene. The standard deviations for PC3-C and PC3-AR-V7 were 0.06 and 0.091, respectively. Only one experiment was performed in quintet.

**Establishment of stable cell line expressing AR-V7 in PC3 cells**

Expression of proteins from transiently transfected cells usually lasts only a few days. Therefore, transient transfections needed to be conducted for each experiment. To avoid the variations induced from different transfections, we decided to establish a cell line stably expressing AR-V7. We obtained the AR-V7-expressing plasmid from Dr. Luo (Johns Hopkins University) in which the AR-V7 open reading frame (ORF) was cloned into the pcDNA.3.1 vector. The Invitrogen pcDNA3.1 protocol suggested that the linearized plasmid would reduce the chances of disrupting key cellular elements. Based on the multiple cloning sites in pcDNA3.1 (Figure 5), we conducted bioinformatics analyses and identified three restriction
sites (EcoR1, Kpn1, and Xho1) as potential digestive enzymes that can be used to linearize the plasmid without digesting AR-V7.

The plasmid was linearized with each of these three enzymes (EcoR1, or Kpn1, or Xho1) and the linearized DNA was separated on 1% agarose gel (Figure 6). Both Xho1 and Kpn1 appeared to successfully linearize the plasmid (lanes 2 and 3). EcoR1 appeared to digest or partially digest the ORF of AR-V7; multiple bands were seen on the gel (lane 1).
The plasmid linearized by Kpn1 or Xho1 was purified as described and used to transfect PC3 cells with different amounts of lipofecamine 2000. The pcDNA3.1 vector encodes the neomycin resistance gene and this enabled selection of the cells transfected with the plasmid (Figure 5). To conduct the selection, the PC3 cells transfected with or without the plasmid were cultured in media containing 100μg/ml of neomycin (a predetermined concentration that kills the PC3 cell in about two to three weeks). As expected, cells without transfection started to die in the neomycin-containing media and all of them died out by the end of the 3rd week. However, the cells transfected with the linearized plasmid survived in the neomycin-containing media and proliferated during the same time period in identical culture conditions. To verify that the AR-V7 was expressed in the pooled stable cell line, western blot assays were conducted with an antibody against the N-terminus of AR (AR-N20). As seen in Figure 7A, AR-V7 was weakly expressed in the pooled stable cell line (lane 1), but not in the parental (lane 2) PC3 cells. In order to
test if the AR-V7 in the pooled stable cell line was functional, total RNA was purified from both the pooled stable cell line as well as the parental PC3 cells. RT-PCR was conducted to estimate the levels of the PSA mRNA. As shown in Figure 7B, PSA mRNA was not detectable in either cell line whereas the GAPDH cDNA was readily amplified in both of them. In addition, both PSA and GAPDH were successfully amplified when the RNAs purified from LNCaP were used in the RT-PCR, suggesting the PCR worked as it should. Therefore, although the AR-V7 was weakly expressed in the pooled stable cell line, it was not as functional as seen in the transiently transfected AR-V7-PC3 cells.

Figure 7: Pooled AR-V7 PC3 stable cell line (A) Whole cell lysates were made from both pooled stable cell line (lane 1) and the parental PC3 cells (lane 2) and 50μg of protein was separated on SDS-PAGE. Separated proteins were transferred to a nitrocellulose membrane and western blots were conducted with an antibody against the amino-terminus of AR (AR-N20). (B) Cells from the pooled stable cell line (lane 1), and parental PC3 cells (lane 2) were collected, RNA was purified, and RT-PCR was conducted to assess PSA mRNA levels (34 cycles). RNA was purified from LNCaP cells and served as a positive control (lane 3). GAPDH served as an internal control (29 Cycles).

Optimization of the transient transfection conditions

Due to poor expression of AR-V7 and its failure to up regulate PSA in the pooled stable cell line; we decided to continue our research by expressing AR-V7 with transient transfection. We transiently transfected PC3 cells with different ratios of
plasmid DNA and lipofectamine 2000 to determine the best conditions of transfection for AR-V7 expression. Twelve micrograms of AR-V7 plasmid DNA was used for 10cm plates and 4μg of AR-V7 plasmid DNA was used to transfect six well plates. Transfected cells were cultured for 24 hours, total RNA was purified and RT-PCR was conducted to assess PSA mRNA levels. As expected, PSA was not detectable in the PC3 cells without transfection (Figure 8, lane 1). However, increasing intensities of the PSA bands reflect the transcriptional activity of the ectopically expressed AR-V7 with increasing lipofectamine concentrations (lanes 2-5). It appears that transfection with 4μL of lipofectamine (lane 5) showed the strongest up-regulation of PSA mRNA. In addition, PSA was only detected in the positive, but not the negative control and comparable levels of GAPDH mRNA were seen throughout the lanes (1-6), indicating equivalent levels of RNA were loaded into the reaction.

Figure 8: Lipofectamine titration RT-PCR: As designated, PC3 cells were transfected with and without AR-V7-expressing plasmid with varying concentrations of lipofectamine and cultured for 24 hours. Cells were collected, total RNA was purified, and RT-PCR was conducted to assess PSA mRNA (34 cycles). RNAs from LNCaP serves as a positive control. RGDD serves as a negative control. GAPDH serves as an internal control (29 cycles).
To further substantiate the findings in the RT-PCR, lysates from the cells under different transfection conditions were analyzed by western blot with antibody against the N-terminus of AR (ARN20). As shown in Figure 9 and consistent with the RT-PCR results above, the highest AR-V7 protein level was seen when 4μl of lipofectamine was used for transfection (lanes 2-5). AR-FL protein from the LNCaP lysate was used as positive control (lane 6) and actin served as the loading control. Although the actin levels reflected unequal loading, the ratios between AR-V7 and actin suggested that AR-V7 was highly expressed when 4μl of lipofectamine was used for the transfection. Therefore, this condition was used in subsequent transient transfections.

![Western Blot Image](image)

**Figure 9:** Lipofectamine titration western blot: As designated, PC3 cells were transfected with and without 4μg AR-V7 plasmid with varying concentrations of lipofectamine and cultured for 24 hours. Cells were collected, lysed, the proteins in the cell lysates were quantified, and 15μg of protein was separated on SDS-PAGE. Proteins were transferred to a nitrocellulose membrane, and western blots were conducted with antibodies against the amino-terminus of AR and Actin respectively.
**RSV’s effect on AR-V7 transcriptional activity**

We now have a system to test if RSV affects AR-V7 transcriptional activity. Four to six hours after transfection the cells were treated with increasing concentrations of RSV for 48 hours. After the treatment, cells were collected, total RNAs were purified and RT-PCR was conducted. As expected, PSA was not detectable in the PC3 cells without transfection (Figure 10, lane 1), but was detectable in the cells transfected with the expressing plasmid (lane 2). The intensities of the bands gradually decreased when the cells were treated with increasing concentrations of RSV (lane 3-6). In addition, the PSA was only amplified in the positive (lane 7), but not the negative control (RGDD, lane 8). Our internal control, GAPDH, remained comparable throughout PC3 and LNCaP cells (lanes 1-7). QPCR results further proved the validity of our RT-PCR results in Figure 11. Further qPCR experiments need to be done to establish statistical significance.

*Figure 10: RSV effect on AR-V7 transcriptional activity RT-PCR: As designated, PC3 cells were transfected with and without AR-V7, treated with different concentrations of RSV, and cultured for 48 hours. LNCaP serves as a positive control (lane 7). RGDD serves as a negative control (lane 8). Total RNA was purified and RT-PCR was conducted to estimate PSA (34 cycles) mRNA. GAPDH serves as an internal control (29 cycles).*
Figure 11: RSV effect on AR-V7 transcriptional activity qPCR: As designated, PC3 cells were transfected with and without AR-V7, treated with different concentrations of RSV, and cultured for 48 hours. Total RNA was purified and qPCR measuring raw relative quantification (RQ) was conducted followed by a delta delta CT study to quantify PSA up-regulation using GAPDH as an internal control. Standard deviations ranged from 0.061-0.344. Only one experiment was performed and it was performed in triplet.

To determine if RSV affects the transcriptional activity of AR-V7 on other AR target genes, RT-PCR was conducted to assess four other AR target gene mRNA levels. The AR target genes used were UNC13, B2M, NDRG1, and ANKH. As Figure 12 depicts, each AR target gene had comparable mRNA levels among PC3 cells (lane 1), AR-V7-expressing cells (lane 2), and AR-V7 expressing RSV-treated PC3 cells (lanes 3-6). Again, the positive control (LNCaP lane 7) showed amplified PSA but the negative control (RGDD lane 8) did not. Our internal control GAPDH was consistent throughout the cells of interest and our positive control (lanes 1-7) legitimizing our results. Collectively this data indicates that RSV inhibits AR-V7 transcriptional up-

![PSA](attachment:image.png)
regulation of PSA, but not the other AR-targets. The non-transfected PC3 cells’ mRNA was not amplified indicating AR is not responsible for their mRNA expression (UNC13, B2M, NDRG1, and ANKH).

![Image of RT-PCR results](image.jpg)

**Figure 12**: RSV effect on AR-V7 transcriptional activity of AR target genes: As designated, PC3 cells were transfected with and without AR-V7, treated with different concentrations of RSV, and cultured for 48 hours. LNCaP serves as a positive control (lane 7). RGDD serves as a negative control (lane 8). Total RNA was purified and RT-PCR was conducted to estimate UNC13, B2M, NDRG1, and ANKH, mRNA levels (29 cycles). GAPDH serves as an internal control (29 cycles).

### RSV’s effect on AR-V7 subcellular location

In order to determine how RSV affects AR-V7 transcriptional activity, we sought to determine if RSV affects AR-V7 subcellular locations. PC3 cells were transfected with AR-V7-expressing plasmid using lipofectamine, and cultured for 48 hours with or without RSV. Whole cell lysate, nuclear, and cytoplasmic extracts were prepared and the proteins were analyzed by western blot. Prior to incubation with antibodies, the membrane was stained with ponceau red which showed total protein
transferred to ensure equal amounts of protein were loaded. As shown in Figure 13A, although the total protein concentrations varied significantly among the total lysates, the nuclear and cytoplasmic extracts had comparable protein levels between treatment groups indicating that RSV did not affect the total proteins in each extract. The intensities of the β-tubulin bands were also comparable between samples treated with or without RSV. In the total and cytoplasmic fractions there was significant cytoplasmic contamination of the untreated nuclear sample as observed by β-tubulin detection (Figure 13B). However, as seen in Figure 13C, lanes 1 and 2, RSV treatment reduced the AR-V7 protein level dramatically in whole cell lysate. The effect of RSV on the AR-V7 cytoplasmic fraction was much less (lanes 3 and 4). Nevertheless, it appears less AR-V7 was in the nucleus when the cells were treated with RSV (lanes 5 and 6).

To explore if RSV affects AR-V7 subcellular location from a different perspective, immunostaining was done. As shown in Figure 14, AR-V7 or AR (in red, top row) was stained with antibody against the N-terminus of the AR or AR-V7. The DNA was stained with hoechst (blue, middle row) to indicate the nuclei and the images were merged (bottom). Figure 14 shows the results of the immunostaining of AR-V7 expressing PC3 cells that were treated with or without 100μM of RSV. LNCaP was also stained as a control and the AR was stained and appeared to be located in the nucleus (right column). It appears that RSV treatment reduced the number of cells (Panel B). The levels of AR-V7 (in red) reduced dramatically when the cells were treated with RSV. Again, the results from this experiment showed dramatic overall
decreased AR-V7 protein in the RSV treated cells. Together with the results obtained from the western blot assays, this data suggests that reduced levels of AR-V7 in the nuclei when the cells were treated with RSV was likely due to the overall reduction of the AR-V7 protein levels.

Figure 13: RSV effect on AR-V7 subcellular location western blot: PC3 cells were transiently transfected with and without AR-V7-expressing plasmid. Cells were treated with and without 100µM of RSV. Whole cell lysates, nuclear and cytoplasmic extracts were analyzed by western blot assays, 73 µg of protein was used. Ponceau red staining was done to visualize the proteins transferred to the nitrocellulose membrane (panel A). Western blots were conducted with antibodies against the amino-terminus of AR (AR-N20) (panel 3) and β-tubulin (panel 2) individually.
RSV down-regulates AR-V7 protein expression

Based on the results described above, we decided to further confirm that RSV down-regulates AR-V7 protein levels by treating the transfected cells with different concentrations of RSV for 48 hours. Transiently transfected AR-V7-expressing PC3 cells were treated with increasing concentrations of RSV and the AR-V7 protein levels were estimated by western blot assays. After transferring, the membrane was stained with ponceau red (Figure 15A) and comparable total proteins were seen in all of the treatments. In addition, the β-tubulin levels were comparable among the
samples treated with different RSV concentrations, albeit higher levels of β-tubulin were seen in the sample without transfection (15B, lanes 1-6). As expected, AR-V7 protein was undetectable in PC3 cells without transfection (Figure 15C, lane 1) and it was well expressed when the cells were transfected with the expression plasmid (Figure 15C, lane 2). Of note, the levels of AR-V7 reduced gradually when the cells were treated with increasing concentrations of RSV (Figure 15, lanes 2-6). In addition, results from semi-quantification of the intensities of the protein bands using image J software indicate RSV down-regulated AR-V7 protein levels in a dose-dependent manner.

**RSV down-regulates AR-V7 post-transcriptionally**

In order to determine the mechanisms for RSV down-regulation of AR-V7 protein and transcriptional activity, we assessed AR-V7 mRNA levels after treating the cells with increasing RSV concentrations. As shown in Figure 16, AR-V7 was not detectable in PC3 AR-V7 naïve cells (Figure 16, lane 1), but the AR-V7 was readily detectable in the transfected cells (lane 2). However, the AR-V7 mRNA was not affected when cells were treated with different concentrations of RSV (Figure 16, lanes 2-6). Of note, the internal control GAPDH showed consistent mRNA levels in all treatments (lanes 1-7). Furthermore, GAPDH, but not AR-V7 mRNA was detectable in LNCaP cells (lane 7) and neither GAPDH nor AR-V7 mRNA was detected in the negative control (RGDD, lane 8), authenticating the RT-PCR. These results demonstrated that RSV did not affect AR-V7 mRNA. Together with the results from the previous section, we conclude that RSV down-regulates AR-V7 post-transcriptionally.
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Figure 15: RSV effect on AR-V7 protein expression: As designated, PC3 cells were transfected with or without AR-V7 expressing plasmid and treated with different concentrations of RSV and cultured for 48 hours. Total cell lysates were collected, and protein was quantified. Proteins from whole cell lysates (60 μg) were separated on SDS-PAGE. Proteins were transferred to a nitrocellulose membrane. Ponceau red stain was added and visualized (Panel A), western blots were conducted with antibodies against AR (panel C) or β-tubulin (panel B). Image J software was used to compare relative densities of AR protein bands to non-transfected PC3 cells. Relative densities were adjusted using relative densities from β-tubulin protein bands.

Figure 16: RSV regulation of AR-V7 post-transcriptional activity: As designated, PC3 cells were transfected with and without AR-V7 expressing plasmid, treated with different concentrations of RSV, and cultured for 48 hours. LNCaP serves as a positive control (lane 7). RGDD serves as a negative control (lane 8). Total RNA was purified and RT-PCR was conducted to estimate AR-V7 (29 cycles) mRNA and GAPDH (29 cycles) mRNA.
Discussion

It has been shown that positive AR-V7 status correlates strongly with both CRPC status and poor prognoses in late stage prostate cancer patients\textsuperscript{39}. More importantly, AR-V7-positive prostate cancers have proven to be resistant to almost all commercially available prostate cancer drugs and therapies\textsuperscript{27,28,30}. It was recently discovered that circulating tumor cells can be taken from CRPC patients and using a single tube assay the two major AR isoforms can be differentiated\textsuperscript{40}. Since the majority of patients with CRPC have AR-V7, it is critical to develop a new class of drugs targeting AR-V7 specifically. Other researchers are currently testing AR-V7 activity using prostate cancer-derived cell lines such as LNCaP, VCaP, and non-prostate cancer-derived cell lines such as COS\textsuperscript{41-43}. LNCaP and VCaP cells are human prostate cancer lines expressing endogenous AR-V7\textsuperscript{44}, although our research conditions did not express AR-V7 in LNCaP cells. LNCaP and VCaP cells also express the full-length AR. Therefore, LNCaP and VCaP are ideal in studying the interactions between AR-V7 and AR-FL, but it is almost impossible to specifically study AR-V7 using these cell lines. According to American type culture collection (ATCC) the COS cell line is derived from the kidney of *Cercopithecus aethiops*. COS cells are easily transfectable and expresses high levels of AR-V7. We believe the PC3 cell line is more ideal to study the effects of RSV on AR-V7 transcriptional activity because PC3 is a prostate cancer-derived cell line and does not express endogenous AR nor AR variants. As depicted in the RT-PCR figures presented herein, PSA was not detectable in any of the PC3 AR-V7 naïve cells; further validating that PC3 does not have basal levels of AR. Therefore, the PC3 cells not only provided a prostate cancer
environment but were completely devoid of the effects of AR and AR-Variants on AR-V7 activity.

After demonstrating that ectopically expressed AR-V7 in PC3 cells was functional in regulating AR target gene PSA, we attempted to use PC3 cells to create a cell line expressing AR-V7 stably. The manufacturer recommended that specifically linearized plasmid be used to reduce the chances of interrupting crucial cellular elements when integrating into the cells. We then tried to purify the linearized plasmid by separating the linearized plasmid on agarose gel. Although the yield of the purification was extremely low (about 0.0862μg/μL) we were able to go through the procedure of stable cell line establishment. The cell line was initiated in a well of a 6-well plate. After transfection and antibiotic selection, it appeared that we had a successful cell line. However, the western blot assays showed barely detectable AR-V7 (Figure 7) and the expressed AR-V7 in the stable cell line failed to regulate AR target gene PSA (Figure 7). According to the diagram provided by the pcDNA3.1 manufacturer (Figure 5) the linearization of the plasmid could have separated the open reading frame from the promoter or the polyadenylation sequence. Disrupting these interactions could lead to decreased AR-V7 expression. Since all of the three enzymes (EcoR1, Kpn1, Xho1) in our lab were old, we decided to linearize the plasmid with all of them individually and hoped some of them would work. All three restriction enzymes were supposed to linearize the plasmid without digesting the AR-V7 ORF. Multiple bands were readily seen when EcoR1 digested the plasmid. Therefore, we only used the DNA linearized by the other two enzymes (Kpn1 or
Xho1) to establish the cell line because they appear to linearize the plasmid without cutting the ORF. Nevertheless, upon closer examination of the linearized DNA, we found a shadow band beneath the DNA linearized by Kpn1 and Xho1, which indicate that these two enzymes may not have worked as expected. In future experiments, we plan to establish a stable cell line without DNA linearization.

After resuming transient transfections, we tested the effect of RSV on AR-V7 transcriptional activity. It was clearly demonstrated that RSV inhibited AR-V7 transcriptional activity in a dose-dependent manner. AR-FL regulates numerous target genes involved in different pathways. In order to determine if AR-V7 functions similarly to AR-FL, we tested four well-established AR target genes, UNC13, B2M, NDRG1, and ANKH. However, none of these genes appeared to be regulated by AR-V7, although it appears that ANKH mRNA was somewhat down regulated by RSV. The bands on the gel in this particular experiment were extremely dark (Figure 12) which leads us to question if the gel ethidium bromide was saturated and there were undetectable differences. This data collectively indicates that although AR-V7 functions similarly to the AR-FL, AR-V7 may potentially have its unique set of target genes, it would be beneficial to repeat this experiment.

To dissect the mechanisms of RSV’s inhibitory effects on AR-V7 transcriptional activity, we first examined the subcellular location of AR-V7 when the cells were treated with RSV. It seems the AR-V7 protein level in the nuclear extract was reduced when the cells were treated with RSV (Figure 13, nuclear extracts).
However, since the total AR-V7 protein levels in the RSV-treated cells were so low (Figure 13, whole cell extracts), it is near impossible to determine if RSV inhibits AR-V7 transcriptional activity through blocking its nuclear translocation. It is important to note that similar results were seen when immunostaining was conducted (Figure 14). It would be interesting to conduct the experiment with a lower concentration of RSV (perhaps 25μM instead of 100μM RSV). In addition, the immunostaining shows that AR-V7 was almost undetectable in most of the cells when they were treated with RSV, but the levels of AR-V7 protein in some RSV-treated cells were similar to that seen in the untreated cells. This suggests that RSV down-regulates the AR-V7 levels in some, but not all of the cells. It would be interesting to explore what caused the AR-V7 to escape RSV regulation in these cells.

The results from the subcellular locations and immunostaining suggest that RSV down-regulates AR-V7 protein level. We then treated AR-V7 expressing cells with increasing concentrations of RSV and the levels of AR-V7 were analyzed by western blot assays. Indeed, RSV down-regulated AR-V7 protein levels in a dose-dependent manner (Figure 15). The CMV promoter controls transcription of AR-V7 in our experiments; the results suggest RSV does not affect transcription from this promoter (Figure 16). These results also suggest that RSV did not affect AR-V7 mRNA stability, which led us to conclude that the effect of RSV on AR-V7 is at the post-transcriptional level. Streicher et al., reported that RSV down-regulates endogenous AR-V7 mRNA through an unknown mechanism45. Given the fact that RSV did not affect the steady state levels of AR-V7 mRNA, it is very unlikely that
RSV-mediated AR-V7 mRNA down regulation is due to inhibition of AR-V7 transcription. Therefore, we speculate that RSV represses AR-V7 transcriptional activity post-transcriptionally in our system.

An interesting new discovery related to our study is nicolsamide’s (a well-studied antihelminthic drug) ability to reduce AR-V7 recruitment to the PSA promoter resulting in decreased PSA transcription. Further exploration demonstrated that Niclosamide utilizes a proteasome-dependent pathway to degrade AR-V7 protein resulting in decreased AR-V7 protein level\(^46\). However, due to nicolsamide’s function as an anti-helmithic drug it has multiple actions unrelated to prostate cancer and a plethora of side effects, making it an unattractive therapeutic option. Comparatively, our studies have shown that RSV functions similarly to nicolsamide in that it reduces AR-V7 transcriptional activity by reducing its protein levels. RSV has almost no side effects and has been shown to have positive effects on skin disorders, type II diabetes, cardiovascular diseases, and obesity (Reviewed by Novelle et al.,\(^47\)). Therefore, RSV would be superior to nicolsamide in counteracting AR-V7 transcriptional activity. However, the drawback to RSV as a potential therapeutic option is its low bioavailability in human tissue\(^48\). A solution to the low tissue bioavailability of RSV could be a chemical known as Pterostilbene (PTER). PTER is a structural analog of RSV with greater tissue bioavailability than RSV\(^49\). Using the model system, we established in this study, we can test PTER’s effects on AR-V7 transcriptional activity. If it affects AR-V7 similarly to RSV, PTER could be an even better chemical to repress AR-V7 transcriptional activity in CRPC. Currently, many
researchers are targeting ways to repress transcription levels of AR-V7. For example, Jin et al., discovered a pathway, NF-kB, which increases AR-V7 expression in prostate cancer cells. They found that when NF-kB signaling pathway is blocked, both AR-V7 mRNA and protein were down regulated. Furthermore, blocking NF-kB signaling makes CRPC cells become ADT-responsive50. Therefore, a combination of therapies targeting AR-V7 mRNA levels (such as blocking NF-kB) with RSV and/or PTER could be more efficacious in the treatment of AR-V7-positive CRPC.

Another interesting chemical we would like to test with this system is metformin. Metformin, a drug used for the treatment of type II diabetes, has shown to have anti-prostate cancer capabilities51. Recently, metformin has been shown to down regulate both AR-V7 and AR-FL in a dose-and-time-dependent manner52. Metformin has been shown to decrease AR/ARE interaction. But the mechanism behind metformin’s repressive effects on AR-V7 isn’t understood yet. Using our system to test metformin’s effect on AR-V7 activity could help to uncover the mechanism behind metformin-mediated AR-V7 down regulation. Furthermore, AR-V7 can hetero-dimerize with AR-FL in the absence of androgens and enhance AR-FL activity24, inhibition of AR-V7 would be expected to hamper AR-FL activity.

In conclusion, we have established a system, which enabled us to demonstrate that RSV inhibits AR-V7 protein levels. Mechanistically, RSV represses AR-V7’s protein levels through post-transcriptional regulation, but without affecting its nuclear translocation. This system could be used to screen other chemicals’ effects on AR-V7.
in a prostate cancer environment independent of AR-FL and other AR variants. Finally, the findings from this research suggest that RSV possesses the potential to become a therapeutic option either by itself or in combination with other drugs for patients with AR-V7-positive CRPC.
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