The Effects of Resveratrol on DBC1 & Cadherin Expression in Prostate Cancer

A Thesis in Biomedical Sciences by Micah Bower-Kaiser

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

August 8, 2019
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Prostate cancer (PCa) is an incredibly common disease in the United States, with approximately 170,000 new diagnoses and 30,000 deaths occurring on an annual basis. The current mainstay of treatment for PCa is known as androgen deprivation therapy (ADT), which has proven to be an effective short-term option in earlier stages of the disease. Unfortunately, longer periods of ADT risk accelerating progression of the cancer to the point where 25% of all treated patients relapse to an incurable, metastatic, and highly lethal form known as castration-resistant prostate cancer (CRPC). In approximately 60% of CRPC cases, a splice variant of androgen receptor called AR-V7 can be detected and has been shown to contribute to this progression.

Recent studies have shown that resveratrol (RSV) is capable of increasing the polyubiquitination and subsequent proteasomal degradation of ARV7 proteins in CRPC cells, though the mechanism behind this effect remains unknown. To investigate the hypothesis that this may occur via downregulation of DBC1 proteins which normally blocks the polyubiquitination site on AR-V7, 22RV1 cells were cultured and treated with varying concentrations of RSV for up to 24 hours. Results showed that RSV post-transcriptionally downregulates levels of DBC1 while reducing levels of AR-V7 proteins and the expression of its target genes. N-cadherin, a known target gene of AR-V7, was also shown to be downregulated by RSV in a statistically significant and dose-dependent manner at the mRNA and protein levels. RSV was shown to simultaneously increase levels of E-cadherin proteins and mRNA in a statistically significant and dose-dependent manner, suggesting it may be capable of inhibiting or preventing the epithelial-to-
mesenchymal transition (EMT) in CRPC cells. Together, these findings implicate RSV as a promising therapeutic option for the majority of CRPC patients who are ARV7-positive.
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<tr>
<td>ADT</td>
<td>androgen deprivation therapy</td>
</tr>
<tr>
<td>APC/C</td>
<td>anaphase-promoting complex/cyclosome</td>
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<td>AR</td>
<td>androgen receptor</td>
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<td>AREs</td>
<td>androgen response elements</td>
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<td>CHX</td>
<td>cycloheximide</td>
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<td>CRPC</td>
<td>castration-resistant prostate cancer</td>
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<td>DBC1</td>
<td>deleted in breast cancer</td>
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<tr>
<td>DBD</td>
<td>DNA-binding domain</td>
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<tr>
<td>DHT</td>
<td>5α-dihydrotestosterone</td>
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<td>EMT</td>
<td>epithelial-to-mesenchymal transition</td>
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<td>mesenchymal-to-epithelial transition</td>
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<td>N-terminal domain</td>
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<td>OS</td>
<td>overall survival rate</td>
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<td>PCa</td>
<td>prostate cancer</td>
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<td>prostate-specific antigen</td>
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<td>reverse-transcription polymerase chain reaction</td>
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<td>relative quantification</td>
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<td>SDS-PAGE</td>
<td>SDS polyacrylamide gel electrophoresis</td>
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INTRODUCTION

1.1 Background: prostate cancer diagnosis & treatment

Prostate cancer (PCa) is an incredibly common disease in the United States, with approximately 170,000 new diagnoses and 30,000 deaths occurring on an annual basis.\(^1\) As the second most common cancer in males, 1 out of every 9 American men will be diagnosed with prostate cancer at some point in their lives and 1 in 41 will die from the disease.\(^1\)

Although the overall rate of prostate cancer in the US is declining, the steady rise in number of diagnoses each year is largely the result of increasing population and improved diagnostic techniques which allow for earlier detection of the disease before symptoms arise.\(^2\) The most common and widely utilized of these techniques is the prostate-specific antigen (PSA) assay. However, earlier detection has not reduced the number of deaths from the disease.\(^2,3\) Though there are a number of factors such as age and growth rate that can influence which treatment options are available to patients, one of the primary determinants is the stage of the cancer at diagnosis (Figure 1).\(^4\)

In the early preclinical stages of prostate cancer, an effective first option for many is watchful waiting, during which a physician will temporarily forgo treatment and closely monitors the cancer’s progression until it is deemed necessary to pursue more aggressive treatments such as surgery or radiotherapy. In some cases of PCa, the cancer can progress so slowly that the patient will never experience any symptoms, and they can forgo treatment altogether. For the remainder of cases, however, the varying risk of permanent side effects such as urinary incontinence or impotence—which largely depend
upon the skill of the surgeon—make these more aggressive options less than ideal.¹,⁴
While the tumor is still confined to the interior of the prostate, radical prostatectomy—a
procedure which involves complete removal of the gland and surrounding tissue—is the
most effective curative treatment available, though hormone therapies remain the primary
treatment option for prostate cancer (Figure 1).

![Figure 1. Standard treatment options by stage in prostate cancer (PCa).](image)

Hormone therapy or androgen deprivation therapy (ADT) is often an effective
short-term option for many with early stage PCa, however prolonged treatment has
shown no association with improved patient outcome and in some cases has even been
found to accelerate progression of the cancer.⁵ As it stands, around 25% of PCa patients
treated with ADT relapse within 3 years to a much more advanced, aggressive, and
metastatic form known as castration-resistant prostate cancer (CRPC).⁶,⁷ There are
currently no reliable curative treatments for prostate cancer once it becomes castration-
resistant and what limited therapeutic options remain such as chemotherapy and
immunotherapy have only proven effective at prolonging life by a few months.⁸ Clearly,
there is a demand for new and better therapeutic options for patients diagnosed with progressive forms of prostate cancer and CRPC.

1.2 Mechanisms of PCa progression & Androgen Deprivation Therapy

Androgen & androgen receptors (AR) play a key role in the growth and maintenance of a healthy prostate, but they can also be among the primary culprits behind the development and progression of prostate cancer. The androgen receptor (AR-FL) is a protein in prostate cells comprised of three primary functional domains (Figure 2). At the N-terminal is a transactivation domain (NTD) which facilitates regulation of canonical AR target genes responsible for cell maintenance, proliferation, and survival. Adjacent to the NTD is the receptor’s primary DNA-binding domain (DBD), followed by a hinge region which allows homodimerization to other AR when activated. Finally, the ligand-binding domain (LBD) at the C-terminus contains the binding site for androgens and will keep the receptor in an inactive state unless the androgen is bound.

![Figure 2. Protein structures and functional domains of AR-FL and AR-V7.](image)

In both the full-length androgen receptor (AR-FL) and AR-V7, functional domains include the n-terminal domain (NTD) responsible for transcriptional activity and DNA-binding domain (DBD) facilitating DNA-protein binding to androgen response elements (AREs). AR-FL contains a hinge region to facilitate homodimerization to AR and a ligand binding domain (LBD) which house the androgen binding site. In AR-V7, both the hinge region and LBD are missing, resulting in androgen-independence.
In healthy prostate tissue, androgen hormones such as testosterone that are synthesized in the testes and adrenal glands will enter cells where they are then converted into a more potent form known as 5α-dihydrotestosterone (DHT). The androgens or DHT can then bind to inactive AR located within the cytoplasm (Figure 3a). Binding of the androgen ligand to AR serves as its activation signal, mobilizing the receptor to dimerize with another activated AR to form the homodimer which can then translocate to the nucleus. Once there, the DBD binds to specific DNA sequences known as androgen response elements (AREs) located within the enhancer/promoter regions of canonical AR target genes. The NTD then recruits various transcription factors and alters the expression of those genes.

One protein produced and secreted by this signaling pathway is prostate-specific antigen (PSA). In the development of PCa, gradual overactivation of AR causes dysregulated growth of cells within the prostate and also increases the expression of PSA. Increased levels of PSA in the bloodstream is one of the primary markers for prostate cancer. If left to progress, this can result in tumorigenesis and eventually spread to other parts of the body. Amplification and/or mutations in the AR gene, overexpression of coactivators, and alternative splicing are all mechanisms by which this gradual AR overactivation can occur.
In general, treatment with androgen deprivation/hormone therapy targets AR and deprives the androgen hormone serving as its activation signal.\textsuperscript{6,10} Enzalutamide and Abiraterone, two of the major drugs used in ADT, function by blocking the hormone binding site on AR itself or by inhibiting the body’s ability to synthesize androgens, respectively (Figure 3b).\textsuperscript{5}

![Figure 3. Androgen/AR signaling in prostate cancer and mechanisms of androgen deprivation therapy (taken from Nelson et al.).\textsuperscript{5} Androgen hormone in prostate cancer cells binds over-activated androgen receptors (AR) causing homodimerization and translocation to the nucleus. Activated nuclear AR homodimers regulate transcriptional activity of the cells, facilitating a dysregulated increase in proliferation, survival, and secretion of prostate-specific antigen (PSA)(A). Androgen deprivation therapy (ADT) restricts cancer cell response to AR overactivation by inhibiting androgen-AR interaction. Enzalutamide and Abiraterone are two major drugs used in ADT which function via competitive inhibition at the androgen binding site or inhibition of the body’s \textit{de novo} synthesis of androgen hormone, respectively (B).]
Eventual progression of prostate cancer most often occurs via upregulation of AR splice variants less dependent on androgen as the body tries to adapt to the stresses imposed by prolonged androgen deprivation. By far the most prevalent of these variations, detected in over 60% of CRPC diagnoses, is known as AR-V7. This mutation generates a truncated but constitutively-active form of the AR that completely lacks the ligand binding domain, allowing it to function without dependence on the androgen hormones targeted by ADT (Figure 2).

Expression of AR-V7 within PCa tissues can be detected by core needle biopsy of the prostate, and has been associated with decreased survival, shorter progression time to CRPC, and higher rate of recurrence even after radical prostatectomy. Overexpression of AR-V7 has also shown to be strongly associated with older age (>70 y/o) at diagnosis meaning that, like prostate cancer, age is a prominent risk factor. One reason for this may be the gradual decline in testosterone synthesis that typically occurs as men age, given that physiological androgen levels show an inverse correlation with AR-V7 expression. Seeing as AR-V7 is detected in a majority of CRPC cases, this would be consistent with other studies which have shown that patients with CRPC who exhibit higher (>5 ng/dl) serum androgen levels are significantly more likely to benefit from ADT than those with lower (<5 ng/dl) levels of androgen.

1.3 Survey of current research and study aims

Recently, a critical coactivator for AR-V7 in CRPC cell lines was identified as deleted in breast cancer (DBC1), an AR-FL cofactor which preferentially associates with AR-V7 and enables it to perform a wide variety of its functions commonly-linked to
CRPC progression and metastasis.\textsuperscript{6} One such function specific to AR-V7 is the selective recruitment to and increased DNA binding affinity for the CDH2 enhancer region upon association with DBC1 (Figure 4). Once AR-V7 binds the enhancer, the associated DBC1 portion undergoes a conformational change and interacts with the CDH2 promoter region \sim 20 \text{ kb} upstream to form a chromatin loop. From this structure, DBC1 facilitates numerous long-distance chromatin interactions which enable and upregulate transcription of CDH2.\textsuperscript{6}

The CDH2 gene encodes the N-cadherin protein that is well-established as one of two primary drivers of the Epithelial-to-Mesenchymal Transition (EMT).\textsuperscript{6,16} This mesenchymal marker of EMT is commonly upregulated in CRPC and other metastatic cancers.\textsuperscript{6} Cancer cells which undergo this transition exhibit increased migratory capacity, invasiveness, and a heightened resistance to apoptosis.\textsuperscript{17} In prostate cancer specifically, the increase in expression of N-cadherin is considered to be one of the major causes of progression to metastasis and the development of castration-resistance in CRPC.\textsuperscript{6,16,18}

Once the transitioned cancer cells have adopted the more mesenchymal phenotype, they can more readily detach from the primary tumor and spread throughout the body. Eventually, they revert to the more adhesive epithelial phenotype through what is known as the mesenchymal-to-epithelial transition (MET) and attach themselves at peripheral sites, where they can begin proliferating to form the secondary tumor.

Although the role of androgens in regulating N-cadherin is not yet known, lower testosterone levels and therapeutic treatment with androgen deprivation has been strongly associated with its upregulation.\textsuperscript{18} In addition to this, N-cadherin/CDH2 expression during the epithelial-to-mesenchymal transition is reciprocally paired to that of CDH1—a
gene which encodes E-cadherin, the other major driver of EMT and the primary cell-cell adhesion molecule in epithelial cells.\textsuperscript{17} Although the upregulation of N-cadherin is still required, downregulation of E-cadherin must also occur before EMT can initiate.\textsuperscript{19}

Like CDH2/N-cadherin, another target gene which is shown to be upregulated by AR-V7, but not the full-length AR, is the ubiquitin-conjugating enzyme E2C (UBE2C).\textsuperscript{20,21} This enzyme within the ubiquitin-proteasome pathway functions by initiating the polyubiquitination and degradation of anaphase-promoting complex/cyclosome (APC/C) substrates which are crucial to proper chromosome segregation and the progression of mitosis.\textsuperscript{22,23} As of 2019, overexpression of UBE2C has been detected in over 27 different forms of cancer.\textsuperscript{23} In PCa specifically, increased expression of UBE2C is directly correlated with increased AR-V7 expression, and has been associated with shorter overall survival (OS) times, poorer clinical outcomes, and can indicate tumor progression.\textsuperscript{21,23} For this reason, it has become a firmly established therapeutic target in the treatment of many different cancers and diseases.\textsuperscript{23}

An additional function of DBC1 is increasing the stability of AR-V7 by occupying the same binding site used by the E3 ubiquitin ligase known as C-terminus of Hsc70-interacting protein (CHIP). In this way, DBC1 effectively competes with the only enzyme currently known to polyubiquitinate AR-V7 and subsequently target it for proteasomal degradation (Figure 4).\textsuperscript{6} Given the enormous prevalence of PCa among men and the relative lack of long-term treatment options for those with CRPC, any methods which can combat the underlying causes of CRPC progression such as AR-V7 or DBC1 are well worth pursuing.
Resveratrol (RSV, trans-3,4,5-trihydroxystilbene) is one such factor investigated for its chemopreventative effects in a wide array of cancers, including PCa.\textsuperscript{24} RSV is a polyphenolic phytoalexin—a compound released by certain plants when under duress—that is most commonly found in red grape skins, blueberries, and even peanuts.\textsuperscript{24} In a recent study, RSV was found to reduce levels of endogenous AR-V7 proteins and the corresponding expression of its target genes without affecting levels of the full-length AR in several different ARV7-positive CRPC cell lines.\textsuperscript{20} RSV was shown to mediate this downregulation in a dose-dependent manner by enhancing the polyubiquitination and subsequent proteasomal degradation of AR-V7 proteins.\textsuperscript{20} The specific nature of the mechanism(s) by which RSV mediates this effect on AR-V7 has yet to be determined.
Given this, and the lack of knowledge regarding the effects of resveratrol on DBC1, I hypothesize that RSV increases polyubiquitination and degradation of AR-V7 by affecting relative levels of CHIP ubiquitin ligase and/or DBC1, which competes with it for binding to AR-V7. If this is determined to be the case, then treatment with resveratrol may also function as a mechanism for decreasing the levels of CDH2/N-cadherin in PCa cells, given that it requires DBC1 for its expression.
MATERIALS & METHODS

Reagents

RPMI (1X) with L-glutamine culture media was purchased from Corning Cellgro and stored at 4°C. Premium Fetal Bovine Serum (FBS) was purchased from Atlanta Biological (Oakwood, GA) and stored at -20°C. Antibiotic-Antimycotic (100X) was purchased from Life Technologies and stored at 4°C. Dulbecco’s phosphate-buffered saline (DPBS) was purchased from Gibco Life Technologies (w/o CaCl/MgCl). Trypsin (0.25%) was purchased from Corning Cellgro and stored at -20°C (without bicarbonate, 0.1% EDTA in HBSS). Resveratrol (RSV) was purchased from Sigma-Aldrich and stored in the dark at -30°C. 200mM stock solutions of RSV were prepared by dissolving resveratrol in 100% EtOH (Biotechnology Grade Anhydrous-Alcohol, IBI Scientific, Peosta, IA). Both the cycloheximide (CHX) solution, and rabbit anti-CHIP antibodies were purchased from Sigma-Aldrich and stored in the dark at -20°C.

From Invitrogen, 0.1M DTT and First-strand buffer (5X) were purchased and stored at -20°C. Both Random 9-mer primers purchased from Agilent Technologies and ROX Reference Dye II purchased from Takara were stored at -20°C. 10 mM dNTP mix and TaqMan qPCR primers for CCAR2, CDH1, CDH2, UBE2C, KLK3, and GAPDH were purchased from Thermo-Fisher Scientific and stored at -20°C.

From Bio-Rad Laboratories, Tris/Glycine/SDS Electrophoresis Buffer (10X), Precision Plus WesternC Protein Standards (stored at -20°C), Precision Protein StrepTactin-HRP Conjugate (stored at 4°C), Clarity and Clarity Max Western ECL
substrates were purchased. Ponceau S Total Protein Stain was purchased from Boston Bio Products. DBC1, ARV7, N-Cadherin, E-Cadherin, and GAPDH antibodies were all purchased from Abcam and stored at -20°C.

**Cell Culture**

22RV1 cells obtained from ATCC Bioproducts were cultured in RPMI 1640 1X with L-glutamine (Corning), 10% premium FBS (Atlanta Biological), and 1% Antibiotic-Antimycotic (Thermo-Fisher Scientific). Cells were grown in either 10 cm culture dishes (CellTreat) with 10 mL of growth media or 6-well culture plates (CellTreat) with 2 mL of growth media replaced every 1-2 days. Cells were kept in incubators at 37°C and 5% CO₂, washed briefly with 1X DPBS (Gibco), and passaged at approximately 70-90% confluence with 0.25% trypsin (Corning). Cells were counted using a Countess II digital cell counter purchased from Life Technologies. Countess cell-counting chamber slides and Trypan Blue stain were purchased from Invitrogen.

**Cell Treatment**

At approximate cell counts of 1 million and 5 million cells, 22RV1 cells were incubated at 37°C and allowed to adhere to 10 cm cell culture dishes or 6-well culture plates, respectively, for 24 hours prior to treatment. Preparation of resveratrol treatments was carried out through serial dilution of 200 mM RSV stock solution to 25 µM, 50 µM, 75 µM, and 100 µM in 1X RPMI 1640 growth media (Corning). Preparation of cycloheximide (CHX) treatments was carried out through serial dilution of 100 mg/mL ready-made cycloheximide solution (Sigma Aldrich, SKU ID# C4859) to 50 nM and 50 µM in 1X RPMI 1640 growth media. Cells were treated for up to 24 hours. Following
treatment, all cells were scraped, collected, and pelleted by centrifuge at 3000 RPM and 1000 RPM. Excess media was aspirated and cell pellets were stored at -30°C prior to RNA purification or preparation of whole cell lysates.

**Total RNA Purification**

RNA from harvested cell pellets was purified using RNeasy Plus Micro Purification Kit (Qiagen) in accordance with manufacturer protocol. RNeasy columns (Qiagen) were used to extract RNA with two elutions of RNase-free H2O (Qiagen) followed by a 1-minute spin at 15,000 RPM after each elution. Total RNA quantification was performed using a NanoDrop 2000c UV-Vis Spectrophotometer purchased from Thermo-Fisher Scientific and samples were stored in the dark at -30°C.

**Reverse Transcription-PCR**

Before cDNA generation, 10 mM dNTPs (Thermo-Fisher Scientific), random-9-mer primers (Agilent), and RNase-free water (Qiagen) were added to 5 µg of sample RNA and incubated in a Techne thermal cycler for 5 minutes at 65°C. Superscript II Reverse Transcriptase Kit (Invitrogen) was then used to generate cDNA in accordance with manufacturer protocol. PCR reactions were carried out in a 3Prime Techne thermal cycler using the following run method: primer annealing (10 minutes at 25°C), primer extension (50 minutes at 42°C), and primer inactivation (15 minutes at 70°C.) 76 µL of nuclease-free H2O (Thermo-Fischer) was added to the resulting cDNA samples which were then stored at -20°C.

**Quantitative Real-Time PCR**
qPCR reactions were carried out using TaqMan Universal PCR kit (Applied Biosystems) in accordance with manufacturer’s protocols. The master mixes were made using [] µg cDNA, RNase-free H2O (Qiagen), TaqMan qPCR premix, ROX II Reference Dye (Thermo-Fisher Scientific), and appropriate qPCR probes. From Thermo-Fisher Scientific, target primers were purchased for CCAR2/DBC1 (human, Primer ID# Hs00377763_CE), CDH1/E-cadherin (human, Primer ID# Hs00345541_CE), CDH2/N-cadherin (human, Primer ID# Hs00174449_CE), and UBE2C (human, Primer ID# Hs00215129_CE) alone or in multiplex with GAPDH (human, Catalog ID# 402869), the housekeeping gene used as an endogenous control. 25 µL of master mix was loaded in triplicate or quadruplicate into each well of a 96-well qPCR reaction plate (Applied Biosystems), capped with 8-strip tube caps (Applied Biosystems), centrifuged for 30 seconds, and analyzed using the StepOne Plus Real-Time PCR System (Applied Biosystems) with the following run method over 40 cycles: hot start (30 seconds at 95°C), denature (5 seconds at 95°C), and extension (37 seconds at 60°C).

**Whole Cell Lysate Preparation & Quantification**

Harvested cells were resuspended in either 50 or 100 µL of radioimmunoprecipitation assay (RIPA) lysis buffer depending on pellet size, vortexed, and left on ice for 15 minutes to lyse. Following lysis, samples were kept at 4°C and centrifuged for 30 minutes at 15,000 RPM. The supernatant lysates were then transferred to new sample tubes and stored at -30°C while remaining cellular debris was discarded. Pierce BCA Protein Assay Kit purchased from Thermo-Fisher Scientific was used in accordance with manufacturer’s protocols to determine total protein concentration of each lysate.
Protein Gel Electrophoresis & Western Blot

Protein samples were prepared using 25-50 µg of total protein, reagent-grade double distilled (RGDD) water, and sodium dodecyl sulfate (SDS) loading buffer. Samples were denatured at 95°C for 5 minutes, then separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (5% stacking, 10% separating) running at 125mV for 2 hours. Following separation, bands were transferred to mini nitrocellulose membranes purchased from Biorad using iBlot gel transfer stacks (Thermo-Fisher Scientific) in accordance with manufacturer protocols. Successful band transfer was verified using Ponceau S total protein stain (Boston Bio Products), which was removed by washing in 1X TBS for 10 minutes.

Membranes were blocked with Fast Western AB Diluent (Thermo-Fisher Scientific) for 1 hour, washed twice with 1X TBS-Tween (0.1%) for 10 minutes each, and incubated overnight in primary antibody dilution at 4°C. Antibodies against ARV7 (rabbit recombinant monoclonal, ID# ab198394), DBC1 (rabbit monoclonal, ID# ab128890), E-cadherin (mouse monoclonal, ID# ab1416), and N-cadherin (rabbit polyclonal, ID# ab76057) were purchased from Abcam and used at 1:1000 dilution, while CHIP (rabbit polyclonal, ID# C9243) was purchased from Sigma-Aldrich and was used at 1:5000 dilution. Antibody against GAPDH (rabbit recombinant monoclonal, ID# ab181602) was also purchased from Abcam and was used as a loading control at a 1:1000 dilution. Membranes were then washed 3x for 10 mins in TBS-T(1X), incubated in secondary antibody conjugated to HRP (Biorad) for 15 minutes at 4°C, washed another 3x for 10 mins in TBS-T(1X), and given a final 10 min wash in TBS(1X). Pierce Fast Western Blot ECL Substrate (stored at 4°C) and Fast Western Optimized HRP Reagent
were purchased from Thermo-Fisher Scientific. Clarity Max Western ECL kit solutions purchased from Biorad were then introduced in a 1:1 mixture to visualize protein bands using a Biorad ChemiDoc MP Imager. Band intensities were quantified and normalized to GAPDH using ImageLab software according to manufacturer protocols (Biorad).

**Statistical Analysis**

SPSS software purchased from IBM was used for statistical analysis of relative quantification (RQ) values in all qPCR experiments. Significant difference among all treatment groups was first determined using one-way ANOVA with an alpha of 0.05. Following statistically significant one-way ANOVA, differences between individual sample groups were then determined using post-hoc Tukey HSD multiple comparisons analysis.
RESULTS

1.1 The effects of resveratrol on the mRNA levels of DBC1

In a previous study, RSV was found to be able to reduce AR-V7 transcriptional activity and the expression of its target genes without impacting levels of AR-V7 mRNA.\textsuperscript{20} As a target gene of AR-V7 but not the full-length AR, expression of UBE2C has also shown to be reduced at both the mRNA and protein levels upon treatment with RSV.\textsuperscript{20,21} What is not yet known is whether DBC1 also exhibits this downregulation under the same conditions and, if so, whether that decrease might play a role in RSV-mediated AR-V7 degradation.

In order to investigate the effects of RSV on DBC1 mRNA, 22RV1 cells expressing high levels of AR-V7 and low levels of AR-FL\textsuperscript{20} were cultured and treated with or without 25-100\(\mu\)M of RSV for a period of 24 hours. qPCR was performed using \(\Delta\Delta CT\) method to measure the relative quantification (RQ) of DBC1 mRNA as the target gene, UBE2C as a positive control, and GAPDH as an internal control.

As seen in figure 5a, UBE2C mRNA levels showed little to no change at 25 \(\mu\)M RSV when compared to the untreated sample but exhibited statistically significant and dose-dependent decreases when treated with concentrations of 50 \(\mu\)M, 75 \(\mu\)M, and 100 \(\mu\)M RSV. This decrease was expected and is consistent with results obtained by Wilson et al.\textsuperscript{20} Additionally, this downregulation is consistent with the previously established RSV-mediated decrease in AR-V7 and its transcriptional activity, suggesting that this effect had successfully been replicated in my experiment.\textsuperscript{20} Under the same conditions, however, mRNA levels of DBC1 exhibited no statistically significant difference across
the four concentrations of RSV when compared to the untreated sample (Figure 5b). This suggests that RSV does not affect DBC1 mRNA levels.

**Figure 5.** RSV induces dose-dependent decrease in UBE2C but does not alter mRNA levels of DBC1.

22RV1 cells were cultured and treated with serial dilution of 25-100µM RSV for 24 hours. qPCR was performed in quadruplicate with GAPDH as endogenous control in multiplex with the target genes. Quantities of target mRNAs relative to untreated controls were calculated by \( \Delta \Delta CT \) method and expressed as mean +/- SEM with C\(_T\) SD <0.1. Graphs show results from three independent experiments (n = 3). Statistical significance was determined by post-hoc Tukey HSD multiple comparisons analysis following statistically significant 1-way ANOVA: *, p<0.05; **, p<0.001. Relative levels of UBE2C mRNA as an established AR-V7 target genes showed significantly decreased levels at 50, 75, and 100µM RSV (A). Relative levels of DBC1 mRNA showed no significant difference across all treatment groups (B).
1.2 Resveratrol down-regulates protein levels of DBC1

Similar to the effects on AR-V7, any resveratrol-mediated downregulation which may occur in DBC1 does not appear to affect its expression at the mRNA level. In AR-V7, however, downregulation was established to occur post-translationally via increased polyubiquitination and subsequent proteasomal degradation. Though the specific mechanisms behind this effect have yet to be discovered, both the downregulation of DBC1 and the upregulation of CHIP remain plausible mechanisms for this increase in AR-V7 polyubiquitination.

To investigate the overall effects of RSV on these two proteins, 22RV1 cells were cultured and treated with or without 100 µM RSV for up to 24 hours and harvested every four hours. Treatment at 100 µM was chosen as it exhibited the greatest downregulatory effect on UBE2C at the mRNA level (Figure 5a), and findings by Wilson et al. using same cell line previously showed significantly decreased AR-V7 proteins at this concentration. Successful gel to membrane transfer was verified using Ponceau S total protein stain as seen in figure 6b, and Western blotting was conducted with antibodies against DBC1, AR-V7, and CHIP as target proteins (Figure 6a). GAPDH served as an internal loading control.

As seen in figures 6a and 6c, DBC1 protein bands in the samples treated with RSV showed a gradual decrease during the first 16 hours post-treatment to around one third of the intensity of the untreated sample. At 20 hours post-treatment, DBC1 bands begin to increase again, ultimately reaching an intensity around 86% of the control at 24 hours (Figure 6c). Together with results from the previous experiment, this overall
decrease in band intensity suggests that treatment with RSV post-transcriptionally downregulates levels of DBC1.

As seen in figures 6a and 6d, AR-V7 protein bands showed a distinctive increase in intensity from the control sample to the first treated sample collected at 4 hours post-treatment, suggesting a brief upregulation of AR-V7 proteins upon initial exposure to RSV. As expected for a positive control and consistent with data obtained in other studies, all subsequent samples collected at 8- to 24-hours post-treatment displayed a gradual decrease in AR-V7 proteins (Figure 6d). After normalization to GAPDH, CHIP protein bands appeared to remain constant in each of the treated samples when compared to those in the untreated sample, suggesting that levels of CHIP proteins are additionally unaffected by RSV (Figures 6e).
Figure 6. Effects of RSV on DBC1, AR-V7, and CHIP protein levels over 24 hours. 22RV1 cells were cultured and treated with or without 100µM RSV for up to 24 hours. Cells were harvested every 4 hours and whole cell lysates were prepared and quantified. 50 µg of total protein per sample was separated via (5%, 10%) SDS-PAGE and transferred to nitrocellulose membranes. Western blotting was conducted using antibodies for DBC1, AR-V7, and CHIP with GAPDH serving as loading control (A). Protein band intensities were normalized and quantified using ImageLab software. Gel to membrane transfer was verified using Ponceau S total protein stain (B). A time-course was plotted showing normalized, relative intensities of DBC1 (C), AR-V7 (D), and CHIP (E) protein bands averaged from three independent experiments, each performed in duplicate (n = 3).
2.1 The effects of resveratrol on DBC1 protein stability

To further investigate the mechanism behind this RSV-induced downregulation of DBC1 proteins, 22RV1 cells were cultured and treated with 50nM or 50µM cycloheximide (CHX) alone or in combination with 100 µM RSV. Cells were harvested at 2-, 4-, 8-, and 12-hours post-treatment, and Western blotting was conducted with antibodies against DBC1 and AR-V7 as target proteins using GAPDH as a loading control (Figure 7).

For the cells treated with 50 nM of cycloheximide seen in figures 7a and 7b, DBC1 protein bands increased in intensity during the first 4 hours post-treatment to approximately 5-times and 3-times the levels seen in the untreated sample for the “CHX + RSV” and “CHX only” groups, respectively. Over the next 8 hours these same groups began to steadily decrease, ultimately reaching levels just above those found in the untreated sample by 12-hours post-treatment (Figure 7b). As seen in figure 7c, the ARV7 protein bands followed a similar trend to DBC1 in both groups, reaching levels about twice those seen in the untreated sample by 12-hours post-treatment.
Figure 7. 50 nM cycloheximide chase analysis showing effects of RSV on DBC1 & ARV7 protein degradation.

22RV1 cells were cultured and treated with 50 nM CHX alone or in combination with 100 µM RSV. Cells were incubated for up to 12 hours and harvested at 2-, 4-, 8-, and 12-hours post-treatment. Whole cell lysates were prepared and quantified using ImageLab. 30 µg of total protein per sample was separated via (5%, 10%) SDS-PAGE and transferred to nitrocellulose membranes. Western blotting was conducted using antibodies for DBC1 and AR-V7 with GAPDH used as loading control (A). Relative protein band intensities were normalized and quantified using ImageLab software. Band intensities for both DBC1 (B) and AR-V7 (C) proteins were averaged between three independent experiments, each performed in duplicate (n = 3), and plotted as a time-course.

When treated with the higher 50 µM concentration of CHX detailed below in figures 8a and 8b, DBC1 proteins in the “CHX only” group showed an immediate decrease to ~60% of the control sample at 2-hours post-treatment. The levels then increased to well above those in the control sample at 4-hours and gradually began
decreasing to ~45% by 12-hours post-treatment. In the group treated with both CHX and RSV, the levels of DBC1 remained relatively unchanged across all 12 hours, the exception being the sample at 4-hours post-treatment which, again, increased to above the levels in the control.

As seen in figure 8c, levels of AR-V7 proteins in both the “CHX only” and “CHX + RSV” groups remained relatively identical to each other at each time interval. As a positive control, this trend is inconsistent with findings obtained under identical experimental parameters by Wilson et al.\textsuperscript{20}, who reported increased AR-V7 degradation in samples treated with RSV. At 8-hours post-treatment, protein levels of AR-V7 in both groups began to decrease steadily, reaching approximately 50% of basal levels by hour 12. Given the inconsistencies to reported findings in other studies and the lack of a viable positive control, only two experiments were run at 50 μM CHX and protein stability results at both concentrations were inconclusive.
Figure 8. 50 μM cycloheximide chase analysis showing effects of RSV on DBC1 & ARV7 protein degradation.

22RV1 cells were cultured and treated with 50 μM CHX alone or in combination with 100μM RSV. Cells were incubated for up to 12 hours and harvested at 2-, 4-, 8-, and 12-hours post-treatment. Whole cell lysates were prepared and quantified using ImageLab. 30 μg of total protein per sample was separated via (5%, 10%) SDS-PAGE and transferred to nitrocellulose membranes. Western blotting was conducted using antibodies for DBC1 and AR-V7 with GAPDH used as loading control. (A). Relative protein band intensities were normalized and quantified using ImageLab software. Band intensities for both DBC1 (B) and AR-V7 (C) proteins were averaged between two independent experiments (n = 2) and plotted as a time-course.

3.1 The effects of resveratrol on the mRNA levels of CDH1 and CDH2

In a recent study, it was shown that DBC1 acts as an essential coactivator required for the expression of many ARV7-specific target genes including CDH2. In addition to this, CDH2 or N-cadherin expression during the epithelial-to-mesenchymal transition (EMT) is reciprocally paired to that of CDH1—a gene which encodes the primary cell-
cell adhesion molecule in epithelial cells known as E-cadherin—though the specific mechanisms behind this link have yet to be discovered.\textsuperscript{17} Although my previous findings have shown that RSV downregulates protein levels of DBC1, it has not been determined whether RSV has any effect on the expression of N-cadherin in prostate cancer cells and if such an effect would be reflected in E-cadherin expression.

In order to investigate the effects of RSV on the mRNA levels of CDH1 and CDH2, 22RV1 cells were again cultured and treated with or without 25-100µM serial dilutions of RSV for a period of 24 hours. qPCR was performed and the housekeeping gene GAPDH was multiplexed with the TaqMan probes for the genes of interest to normalize mRNA expression (Figure 9). As seen in figure 9a, mRNA levels of CDH2 exhibited a statistically significant and dose-dependent decrease at 25, 50, 75, and 100 µM RSV when compared to those in the untreated sample (p < 0.01). This indicates that treatment with RSV downregulates expression of CDH2 at the mRNA level.

Under the same conditions seen in figure 9b, mRNA levels of CDH1 followed a reciprocal trend to that of CDH2, exhibiting a dose-dependent increase with increasing concentrations of RSV. Unlike CDH2, however, mRNA levels of CDH1 were only shown to be significantly different from the untreated sample at concentrations of 50, 75, and 100 µM RSV. This suggests RSV is capable of regulating both CDH1 and CDH2 mRNA levels. Additionally, morphological changes in the cells could be observed at RSV concentrations of 50 µM and higher. With increasing concentration, the number of
cellular extensions and overall size of the cells showed a marked decrease, while overall cytoplasmic granularity, cell detachment, and cell death appeared to an increasing degree.

Figure 9. Effect of increasing concentrations of RSV on CDH1 & CDH2 mRNA levels. 22RV1 cells were cultured and treated with serial dilution of 25-100µM RSV for 24 hours. qPCR was performed in quadruplicate with GAPDH as endogenous control in multiplex with the target genes. Quantities of target mRNAs relative to untreated controls were calculated by ΔΔCT method and expressed as mean +/- SEM with C_T SD <0.1. Graphs show results from three independent experiments (n = 3). Statistical significance was determined by post-hoc Tukey HSD multiple comparisons analysis following statistically significant 1-way ANOVA: *, p<0.01; **, p<0.001. Relative levels of CDH2 mRNA showing significant decreases in all treatment groups when compared with the control group (A). Relative levels of CDH1 mRNA as showing significantly increased levels at 50, 75, and 100µM RSV (B).
3.2 RSV effects on EMT-associated E- & N-cadherin proteins

In order to investigate whether the effects of RSV on CDH2 and CDH1 mRNA are reflected at the protein level, 22RV1 cells were again treated with or without 100 µM RSV for up to 24 hours and harvested every four hours. Successful gel to membrane transfer was verified using Ponceau S total protein stain seen in figure 10b, and Western blotting was conducted with antibodies against both N-cadherin and E-cadherin, using GAPDH as a loading control.

As seen in figures 10a and 10c, N-cadherin protein levels remained relatively unchanged during the first 12 hours post-treatment with RSV. Consistent with the previously observed downregulation of CDH2 mRNA levels, protein levels of N-cadherin at 16- and 20-hours post treatment showed a gradual decline with approximately 50% decrease in intensity by 24 hours when compared to the untreated sample (Figure 10c). This indicates that treatment with RSV downregulates N-cadherin at the both the protein and mRNA levels.

Also consistent with previous results showing upregulation at the mRNA level, protein levels of E-cadherin exhibited a marked increase at 4-hours post-treatment and continued to increase to approximately 2.5 times the basal levels at hour 16 (Figure 10d). By 20-hours post-treatment, protein levels begin to decrease again, though they remain approximately 1.5 times basal levels by hour 24. This indicates RSV upregulates E-cadherin at both the mRNA and protein levels while downregulating levels of N-cadherin mRNA and proteins. As observed in the previous experiment, a dramatic change in cell morphology also began to occur with increasing incubation periods in the treatment media. Slight cytoplasmic granulation and reduction in size appeared to begin at around
8-hours post-treatment with a marked increase in plate detachment and cell death occurring by hour 12.

**Figure 10.** Effects of RSV on N-cadherin & E-cadherin protein levels over 24 hours. 22RV1 cells were cultured and treated with or without 100µM RSV for up to 24 hours. Cells were harvested every 4 hours and whole cell lysates were prepared and quantified. Protein band intensity was averaged from 3 independent experiments (n = 3). 50 µg of total protein per sample was separated via SDS-PAGE and transferred to nitrocellulose membranes. Western blotting was conducted using antibodies for N-cadherin and E-cadherin with GAPDH serving as loading control (A). Gel to membrane transfer was verified using Ponceau S total protein stain (B). A time-course was plotted showing normalized, relative intensities of N-cadherin (C) and E-cadherin (D) protein bands averaged from three independent experiments, each performed in duplicate (n = 3).
DISCUSSION

As of 2019, there are still no reliable curative treatments available for patients diagnosed with CRPC. In over 90% of cases, development of castration-resistance in prostate cancer is accompanied by a shift to a more aggressive and metastatic cancer cell behavior which is highly lethal and quickly spreads to other areas of the body. The most common cause of progression to CRPC detected in over 60% of cases is upregulation of AR-V7, which does not respond to any form of hormone therapy currently used and has been associated with decreased patient survival and shorter progression time to CRPC. Clearly for the many patients who are ARV7-positive and those with CRPC, there is an urgent need for novel treatment options which do not rely upon androgen deprivation and can specifically target AR-V7.

One such compound with the potential to target AR-V7 is resveratrol, which was previously shown to reduce AR-V7 protein levels in prostate cancer cells by enhancing its polyubiquitination and subsequent proteasomal degradation. Within this study, I explored potential mechanisms for this increased polyubiquitination of ARV7 by RSV. Specifically, my investigation focused on CHIP and DBC1, as well as E- and N-cadherins given that they have been implicated in recent studies to play a role in the degradation of AR-V7 and the epithelial-to-mesenchymal transition, respectively.

Given the lack of knowledge regarding the effect of resveratrol on DBC1, I hypothesized that RSV may increase polyubiquitination and degradation of AR-V7 by affecting relative levels of CHIP ubiquitin ligase and/or DBC1, which competes with it for binding to AR-V7. Following from this, I also hypothesized that treatment with
RSV would decrease levels of CDH2/N-cadherin in PCa cells, given that it is a target gene of AR-V7 and requires DBC1 for its expression. Furthermore, the fact that CDH1/E-cadherin expression is inversely tied to that of N-cadherin during EMT lead me to surmise that E-cadherin levels should also increase as a result of resveratrol treatment.

Any potential findings which could be linked to regulation of EMT in PCa are particularly worthy of investigating, as this process has proven to be crucial to cancer cell metastasis and progression to CRPC. So as to establish cellular conditions in vitro which closely approximate most clinical cases of CRPC, my research was conducted using 22RV1 cells—a cell line comprised of human CRPC cells which endogenously express both high levels of AR-V7 and low levels of AR-FL.

In order to determine whether treatment with resveratrol affects the expression of DBC1, I first looked for potential regulation occurring at the mRNA level. To this end, 22RV1 cells were treated with serial dilutions of RSV ranging from 0 – 100 µM and incubated for a period of 24 hours. For UBE2C, a target gene of AR-V7 and not AR-FL acting as the positive control, (Figure 5a) results showed a statistically-significant and dose-dependent reduction in mRNA levels with increasing RSV concentration. This was consistent with findings made in another study and suggests that the RSV-mediated decrease of ARV7 proteins had been replicated in my experiment. As mentioned previously, UBE2C is commonly dysregulated to increased levels in the advanced stages of over 27 different forms of cancer. In PCa, increased expression of UBE2C is directly correlated with increased AR-V7 expression and has been associated with shorter overall survival (OS) times, poorer clinical outcomes, and can indicate tumor
progression. The reduction in UBE2C mRNA levels after RSV treatment shown in this study may provide a relevant avenue for future research as it is a therapeutic target in many different cancers. Results seen in figure 5b showed that under the same conditions, mRNA levels of DBC1 were unaffected by treatment with RSV. These findings suggest that the increased polyubiquitination and degradation of AR-V7 induced by resveratrol are likely not the result of downregulation of DBC1 occurring at the mRNA level.

Given that CHIP upregulation or DBC1 downregulation could both potentially result in enhanced polyubiquitination of AR-V7, I investigated the effects of RSV on protein levels of DBC1, CHIP, and AR-V7 (Figure 6). To this end, 22RV1 cells were treated with or without 100 µM RSV and collected every 4 hours to monitor incubation periods for up to 24 hours. Results from western blotting experiments (Figure 6c) showed that DBC1 protein levels decrease steadily over time with RSV treatment. Somewhat unexpectedly, DBC1 levels did begin to increase again at 20- and 24-hours post treatment, though not to baseline levels found in the untreated sample. One potential explanation for this may be the degradation of resveratrol within the treatment media after ~20 hours. Growth media used to prepare treatments in this study was kept at physiological pH of 7.4, and research on the chemical stability of resveratrol under in vitro conditions has shown that degradation occurs exponentially at pH > 6.8. This is consistent with the fact that the relative decrease in DBC1 protein levels is greatest at 4-hours post-treatment and appears to drop off with time. Unfortunately, this relative instability at physiological pH also demonstrates what is currently one of the major obstacles to the use of RSV as a potential treatment. Not only is it chemically unstable in
conditions within the body, but like many phytochemicals, RSV has a very low bioavailability in human tissues and would require constant administration in high doses to maintain therapeutic concentrations.\textsuperscript{29,30} While RSV is rapidly absorbed when administered orally, it is quickly conjugated and metabolized by the first-pass effect and only trace amounts of unaltered RSV have been shown to make it into systemic tissues.\textsuperscript{29,31} In an effort to combat this problem, a synthetic derivative of RSV known as pterostilbene (PTS) has been developed which, in several studies, has been shown to exhibit both higher bioavailability and stronger pharmacological activity than RSV.\textsuperscript{29,30,32}

As expected in the positive control, protein levels of AR-V7 showed a steady decrease over time with RSV, but only after an initial increase was observed immediately following treatment (Figure 6d). Aside from this increase, these results were consistent with those obtained in a separate study which used the same cell line.\textsuperscript{20} A survey of the relevant literature revealed no functions or interactions which might explain the initial upregulation of AR-V7 proteins upon treatment with RSV, however the regulatory mechanisms which drive the alternative splicing and production of AR-V7 are not currently well understood. In figure 6e it was also shown that levels of CHIP protein remain constant in the presence of RSV. Together, these results indicate that RSV-mediated downregulation also occurs post-transcriptionally in DBC1, albeit with an earlier time of onset by a few hours. Concurrently, levels of CHIP protein appeared to be unaffected by treatment with RSV. This further supports my hypothesis that RSV increases AR-V7 polyubiquitination via downregulation of DBC1 proteins and not via upregulation of CHIP. Though all data gathered over the course of my investigation supported and was consistent with this hypothesis, further research would be required to
state conclusively that this is the case or that it is the sole mechanism involved. With the exceedingly wide range of anti-cancer effects attributed to RSV across a variety of different cancers, it is well within the realm of possibility that more than one of them could be contributing to the increased polyubiquitination of AR-V7.\textsuperscript{24,29,31,33-35}

One major limitation in my study occurred when attempting to further investigate the mechanism for RSV-induced downregulation of DBC1 protein. In using cycloheximide—a potent inhibitor of protein synthesis in eukaryotic cells—to track protein degradation over time, I was ultimately unable to determine whether RSV has any effects on DBC1 or AR-V7 protein stability. Results at 50 nM CHX (Figures 7b-c) showed DBC1 proteins increasing over time and AR-V7 proteins following a similar trend to DBC1 in both groups, reaching levels about twice those seen in the untreated sample by 12-hours post-treatment. Because this was inconsistent with results obtained in previous experiments and seemed to indicate an increase in protein levels following treatment with a protein synthesis inhibitor, the experiment was deemed inconclusive. Given that the EC\textsubscript{50} for CHX to inhibit protein synthesis can vary depending on cell-type and the specific conditions \textit{in vitro}, the experiment was re-attempted at a higher concentration of CHX. Results at 50 \textmu M CHX (Figures 8b and 8c) showed DBC1 seemingly unaffected over time and levels of AR-V7 in both the “CHX only” and “CHX + RSV” groups remaining relatively identical to each other at each time interval. As the positive control, this trend is inconsistent with findings obtained under identical experimental parameters in Wilson et al.\textsuperscript{20}, who reported increased AR-V7 degradation in samples treated with RSV. \textsuperscript{20} Also inconsistent with that study, which showed a 50% reduction in AR-V7 by 2-hours post-treatment, AR-V7 levels in my experiment remained
roughly equal to that of the control sample until hour 8, when they began a gradual decrease and did not reach ~50% until 12-hours post-treatment. Given these inconsistencies with the literature at both concentrations and the lack of viable positive control data, only two experiments were run at 50 µM CHX and I was unable to draw any conclusions regarding the effects of RSV on DBC1 or AR-V7 protein stability. One potential explanation for these inconsistencies is that RSV somehow interferes with cycloheximide’s ability to block protein synthesis. In at least one other study, treatment with RSV in vitro was shown to greatly reduce cycloheximide-induced cell death resulting from inhibited protein synthesis. Another factor which may have contributed to the inconsistency is the slight overlap of the protein bands on the blot, which made it difficult to consistently and reliably quantify individual bands.

CDH2 or N-cadherin expression during the epithelial-to-mesenchymal transition is reciprocally paired to that of CDH1—a gene which encodes the primary cell-cell adhesion molecule in epithelial cells known as E-cadherin. Finding potential cancer treatments which can affect these EMT marker molecules are particularly important given that, alongside the loss of E-cadherin, increased levels of N-cadherin have shown to be one of the primary drivers of progression to metastasis and the development of castration-resistance in PCa. In order to investigate the effects of RSV on mRNA levels of CDH1 (E-cadherin) and CDH2 (N-cadherin), 22RV1 cells were treated with serial dilutions of RSV ranging from 0 – 100 µM and incubated for a period of 24 hours. qPCR results (Figure 9a) showed a statistically significant and dose-dependent decrease in levels of CDH2 mRNA with increasing concentrations of RSV. This suggests that RSV downregulates CDH2 expression at the mRNA level. Consistent with this finding
and the known reciprocal expression patterns of CDH1 and CDH2, results (Figure 9b) showed that levels of CDH1 mRNA were increased in a statistically significant and dose-dependent manner with RSV concentration. This indicates that RSV can both downregulate levels of CDH2 mRNA and upregulate levels of CDH1 mRNA. Having previously established a decrease in DBC1 protein levels with RSV treatment, these findings support my hypothesis that RSV-induced depletion of DBC1 and a resulting reduction in AR-V7 proteins would lead to decreased levels of N-cadherin. More importantly, it suggests that RSV is capable of decreasing the expression of N-cadherin while simultaneously increasing expression of E-cadherin. Given that basal levels of CDH1 were greater than CDH2 in these cells both before and after treatment, this indicates that RSV may be inhibiting the cadherin switch that makes up the crucial first step in the initiation of EMT.\textsuperscript{17,19,37}

In order to determine whether the effects of RSV impact protein levels of N- and E-cadherin, 22RV1 cells were again treated with or without 100 μM RSV and collected every 4 hours to monitor incubation periods for up to 24 hours (Figure 10a). Western blotting results (Figure 10c) showed that protein levels of N-cadherin remain steady until around 16-hours post-treatment with RSV, at which point they begin to decrease steadily. This delayed but present reduction in N-cadherin proteins is consistent with the observed decrease in mRNA levels of CDH2, given the additional time it would take for transcriptional changes to be reflected at the protein level. Together, this shows that treatment with RSV can downregulate N-cadherin expression at both the protein and mRNA levels. This is particularly relevant given that, alongside the loss of E-cadherin,
increased expression of N-cadherin is one of the primary drivers of metastasis and the development of castration-resistance in CRPC.\textsuperscript{16,18}

Results (Figure 10d) additionally showed an immediate and marked increase in E-cadherin proteins after only 4 hours of RSV exposure. These levels continued to increase over the next 12 hours, albeit more gradually, before reaching peak levels at around 16-hours post-treatment. This was consistent with the previously shown increase in CDH1 mRNA and indicates that RSV can also upregulate expression of E-cadherin at both the mRNA and protein levels. The large difference in onset time for this effect when compared to N-cadherin, however, implies that RSV may mediate its effect(s) on E-cadherin through another mechanism. While an upregulation of E-cadherin at the mRNA level is present as well, the strength and onset of the protein increase at 4-hours post-treatment (Figure 10d) may imply the existence of additional upregulatory effects which act on multiple levels. In several other late-stage cancers such as gastric, colorectal, and lung cancer, numerous studies have shown that RSV is capable of increasing E-cadherin at both the mRNA and protein level via downregulation of SNAI1, a nuclear transcription factor which inhibits E-cadherin expression during EMT.\textsuperscript{33,35} In these studies, it was reported that this increase in E-cadherin was also accompanied by significant changes in cell morphology and suppression of the EMT phenotype.\textsuperscript{33,35} This is consistent with the morphological changes in the CRPC cells that were observed in my experiment. There are, however, additional protein markers which characterize EMT that require investigating to conclude for certain if RSV can affect the process itself.

Similar to the levels of DBC1 protein, E-cadherin proteins also appeared to begin decreasing between 16- and 20-hours post-treatment, though they remain at increased
levels when compared to the untreated sample at 24-hours post-treatment. This is consistent with my hypothesis that RSV treatment reciprocally affects levels of N- and E-cadherin, and supports the likelihood that the increase in DBC1 proteins after ~20 hours (Figure 6c) is the result of RSV degradation in the growth media.

This effect is nevertheless relevant, as the loss of E-cadherins and epithelial cell-to-cell adhesion is what is primarily characterized by the epithelial-to-mesenchymal transition, and cancer cells that undergo this transition show significantly increased migratory capacity, invasiveness, and resistance to apoptosis.¹⁷,¹⁸ Having established that downregulation of DBC1 proteins does occur with RSV treatment, these findings lend further support to my hypothesis that RSV-induced depletion of DBC1 and the resulting reduction in AR-V7 proteins may decrease the expression of N-cadherin and increase the expression of E-cadherin. As stated previously, this effect holds the potential to be highly relevant for future studies researching treatment of ARV7-positive CRPC as it suggests RSV may be able inhibit the cadherin switch during EMT that is required for cancer cells to gain their metastatic potential. Because the basal levels of E-cadherin still exceeded those of N-cadherin in the cancer cells in this study, they are still characteristically epithelial and therefore this effect represents a potential inhibition of EMT rather than induction of MET. In any case, the importance of developing the kinds treatments which reduce metastatic potential cannot be overstated as currently over 90% of mortalities linked to any form of cancer arise due to the metastasis and growth of secondary tumors.³⁴

Aside from the inconclusive results regarding DBC1 and AR-V7 protein degradation, one more major limitation/potential source of uncertainty was encountered
during the course of this study. In performing western blots measuring protein levels of DBC1, three distinct signal bands at ~88, 70, and 60 kDa were consistently detected on the membranes when only one is reported in MCF7 cells by the manufacturer of the primary antibody (Figure 11). There are no currently known isoforms or splice-variants of DBC1, so only the strongest signal present at the reported molecular weight of 88 kDa was quantified and reported in this study. Given that the other bands appeared at lower molecular weights, they could represent artifacts of DBC1 degradation, however this typically presents as one long smeared band these appear as discrete and separate signals. Furthermore, degradation product signals would typically reflect the intensity of the primary band, yet expression trends for these are consistent and distinct from those of the primary band or the other signal, so I believe this explanation to be unlikely. It is also possible that they result from overloading the gel with too much protein at 50 µg, however they were still present when 20 and 30 µg of protein were loaded, so this explanation is also not very likely. They are detected by other researchers using 22RV1 cells in the same lab and MCF7 cell lysates run in the same gel show only the primary band, so it is not likely due to personal laboratory technique and may simply be an effect specific to the 22RV1 cell line.

Figure 11. Western blot showing triple DBC1 protein bands at 20 and 50µg in 22RV1 cells treated w/ or w/o 100 µM RSV.
In conclusion, this study has demonstrated in prostate cancer cells that treatment with resveratrol post-transcriptionally downregulates protein levels of DBC1 while reducing levels of AR-V7 protein and the subsequent expression of its target genes. Protein levels of CHIP ligase were unaffected by treatment with RSV, suggesting that it may mediate enhanced AR-V7 polyubiquitination and proteasomal degradation by decreasing levels of DBC1. It was demonstrated that RSV treatment causes downregulation of N-cadherin mRNA and protein levels in a statistically significant and dose-dependent manner. RSV was also shown to simultaneously increase levels of E-cadherin mRNA and proteins in a statistically significant and dose-dependent manner, suggesting it may be capable of inhibiting the epithelial-to-mesenchymal transition in CRPC cells. Together, these findings have indicated that RSV is worthy of further study as a promising therapeutic option for patients diagnosed with ARV7-positive prostate cancer and CRPC.
REFERENCES


