THE ANDROGEN RECEPTOR SPlice VARIANT, ARV7, IN CASTRATIoN-
RESISTANT PROSTATE CANCER:
MOLECULAR ORIGINS, BIPHASIC GROWTH REGULATION, AND RESISTANCE

by
A. Seun Ajiboye

A dissertation submitted to Johns Hopkins University in conformity with the requirements for the
degree of Doctor of Philosophy

Baltimore, MD
March 5, 2016

© 2016 A. Seun Ajiboye
All Rights Reserved
ABSTRACT

Prostate cancer is one of the most frequently diagnosed cancers in the United States and in the world. Advanced prostate cancer is treated by targeting the androgen receptor signaling axis by surgical or pharmacological castration, which is initially an effective treatment strategy. However, all cancer will eventually progress to a castration-resistant state. Castration-resistant prostate cancer is a lethal disease characterized by continued androgen receptor signaling in a castrate setting. New and more potent antagonists to androgen signaling such as enzalutamide and abiraterone were highly successful in clinical trials, but clinical resistance to these treatments is being observed. There are many mechanisms of resistance. The research summarized in this dissertation is focused on enzalutamide resistance mediated by the androgen receptor splice variant, ARV7. We use molecular and cellular biology techniques, including reporter assays, quantitative reverse transcription polymerase chain reaction, immunoblot, and growth assays to understand the molecular origin of ARV7, its mechanism of action, and its role in resistance of several commonly used prostate cancer cell lines. Our results show that ARV7 expression is not a failure of mRNA surveillance, that ARV7 regulates growth and expression of some genes biphasically like the canonical AR receptor, and that the consequences of ARV7 expression on growth is cell-line dependent.

Advisor
Samuel R. Denmeade, MD

Dissertation Readers
Samuel R. Denmeade, MD
Jun Luo, PhD

Dissertation Non-readers:
Srinivasan Yegnasubramanian, PhD
Jun O Liu, PhD
James C. Barrow, PhD
ACKNOWLEDGEMENTS

I would like to acknowledge several people and groups without whom completion of my PhD would not have been possible.

First, I would like to thank Dr. Samuel Denmeade for accepting me into his lab and for his continued support, encouragement, and positive disposition when I was most discouraged. I would also like to thank him for his kindness, understanding, sympathy, the time he took to explain various medical reports to me, and the latitude to visit home as needed when my father was diagnosed with colon cancer.

I would like to thank Dr. Simon Williams, who was a postdoctoral fellow in Dr. Denmeade’s lab I joined as a rotation student. I would like to thank him for the time he took in training me as a young graduate student, for his encouragement, and friendship.

I would like to thank the rest of Sam’s lab, both past and current members (Marc Rosen, Oliver Rogers, Emmanuel Akinboye, PhD, Nate Brennan, PhD, and Maya Kostova, PhD) for their assistance in various aspects of this project, keeping the lab running, and providing a collegial and fun environment in which to do research.

I would acknowledge Isaacs’ lab. In particular, I would like to thank John Isaacs, PhD for sharing his expertise and 30 years of experience on all things related to the androgen receptor and for making me a better scientist by challenging me during lab meetings. I would like to thank Sue Dalrymple and Lizy Antony for sharing reagents and technical expertise with me and for their patience and availability.

I would like to thank William G. Nelson’s lab, the members of which were instrumental in the final stages of this dissertation. In particular, I would like to acknowledge David Esopi, who is a careful, stellar, and patient scientist who taught me about qRT-PCR. I would like to thank Michael Haffner, MD, PhD for his patience and availability in helping with an
immunofluorescence assay, which unfortunately did not make it into this dissertation. I would also like to thank Ajay Vaghasia and Hugh Giovinazzo for their friendship and career advice.

I would also like to thank the members of the Hammers and Kachhap labs for help in providing various reagents and expertise, especially Huong Nguyen who helped me when all my PCR was contaminated.

I would to thank the lab of Dr. Oliver Mühlemann for providing reagents and expertise in for the nonsense-mediated decay assay and Ada Tam and Lee Blosser for helping me to execute the assay.

I would also like to thank the Pharmacology administrators – particularly Mimi Guercio and Amy Paronto – for handling the important administrative tasks so that I could focus on research as well as various faculty members – particularly Drs. Hendrix, Bumpus, and Rudek – for their career advice.

I would like to thank the various friends that I have made in Baltimore for making this a richer experience, for helping me maintain my sanity, and adding more meaning to my life than just work can bring.

Lastly, I would like to thank my family for their continued encouragement, prayer, and support. In particular, I would like to extend my most heartfelt and since gratitude to my parents, Abel and Mary Ajiboye, for the courage and selflessness they showed in coming to America to provide better opportunities for their children, for their many sacrifices while raising us, for putting us all on a path toward success, and for their constant encouragement, belief in my abilities, and unwavering love.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>iii</td>
</tr>
<tr>
<td>List of Tables</td>
<td>vi</td>
</tr>
<tr>
<td>List of Figures</td>
<td>vii</td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Androgen receptor splice variants are not substrates of nonsense-mediated decay</td>
<td>23</td>
</tr>
<tr>
<td>The androgen receptor splice variant, ARV7, in biphasic androgen response</td>
<td>39</td>
</tr>
<tr>
<td>Conclusion</td>
<td>67</td>
</tr>
<tr>
<td>Curriculum Vitae</td>
<td>69</td>
</tr>
</tbody>
</table>
LIST OF TABLES
Introduction

Table 1. Prostate cancer treatment strategies according to stage. 8
LIST OF FIGURES

Chapter 1. Introduction

Figure 1. The prostate in the male reproductive system. 2
Figure 2. Diagram of the Zones of the Prostate. 3

Chapter 2. Androgen receptor splice variants are not substrates of nonsense-mediated decay

Figure 1. Nonsense-mediated decay in 22Rv1 cells. 28
Figure 2. Nonsense-mediated decay regulation of ARV3 and ARV7. 29
Figure 3. Androgen regulation of AR, ARV7, and UPF1 expression 30
Figure 4. Expression of UPF1 in prostate cancer cell lines. 31
Figure 5. Linear Regression Analysis of ARV3 and ARV7 v. UPF1 33

Chapter 3. The androgen receptor splice variant, ARV7, in biphasic androgen response

Figure 1. Androgen-regulated growth of Prostate Cancer Cells in Androgen-depleted and Replete media 45
Figure 2. ARV7-mediated growth inhibition of LNCaP in CSS and FBS 46
Figure 3. Androgen-Regulated Expression of PSA and M-phase genes in CSS and FBS 47
Figure 4. ARV7-regulated Expression of PSA and M-phase genes in CSS and FBS. 48
Figure 5. Partial knockdown of AR abrogates androgen-mediated growth inhibition in FBS. 50
Figure 6. Overexpression of AR abolishes stimulatory effect of androgen on LAPC4 growth 51
Figure 7. Castration-resistant growth of LAPC4-cr.

Figure 8. Castration-resistance of LN95 is not mediated by ARV7.

Figure 9. Castration-resistant 22Rv1 are resensitized by ARV7 inhibition but restimulated by activation of GR pathway.

Figure 10. ARV7-mediated gene expression in 22Rv1 in CSS and FBS.

Figure 11. ARV7 inhibition in 22Rv1 significantly affects growth in FBS.

Figure 12. Comparison of AR and ARV7 protein expression in 22Rv1, LN95, and VCaP
CHAPTER 1. INTRODUCTION

Prostate cancer (PCa) is the fourth most commonly diagnosed cancer behind lung, breast, and bowel cancers worldwide. It is the third most diagnosed cancer behind breast and lung cancers in the United States, where it is estimated that in 2015 over 220,000 men will be newly diagnosed and over 27,500 men will die of the disease, accounting for 13.3% of all new cancer cases and 4.7% of all cancer deaths. The incidence rates of prostate cancer vary by geographical region. However, African-American men have the highest incidence of prostate cancer in the world. The incidence of PCa has decreased since the 1990s and is currently stable. Early diagnosis is critical to long-term survival. Overall, the 5-year survival rate for treated PCa is 98.9%. This number is largely comprised of men who were diagnosed with local or regional disease, who have 100% 5-year survival rate. The 5-year survival rate for men diagnosed with distant metastases drops dramatically to 28.2%.

This chapter will provide an overview of the anatomy of the prostate, the pathophysiology of PCa, and current treatment strategies. It also includes an in depth discussion about therapeutic targeting of the androgen receptor (AR) and mechanisms of drug resistance with special attention to resistance mediated by the AR splice variant, ARV7. This chapter will conclude with a preview of the research detailed in this dissertation.

NORMAL PROSTATE STRUCTURE AND FUNCTION

The prostate is a glandular organ approximately the size of a walnut that is located below the bladder and surrounds the urethra (Figure 1). The prostate is composed of the transitional, central, and peripheral zones, which surround the urethra in layers, in that order (Figure 2). The transitional zone serves a sphincteric function, whereas the central and peripheral zones are responsible for the secretory function of the prostate. Benign prostatic hyperplasia (BPH), a disease that develops in older men and causes lower urinary tract symptoms, occurs in the transitional zone. PCa primarily develops in the peripheral zone.
The primary function of the prostate is to aid in the production of semen, which is a mixture of prostatic, seminal vesicle (posterior to the prostate), and bulbourethral gland (located just below the prostate) fluid and sperm from the testes. Smooth muscles cells in the basement membrane within the prostate gland generate contractile forces to ensure forceful expulsion of semen during ejaculation. Prostate specific antigen (PSA), which is a PCa biomarker and is discussed below, is a serine protease that liquefies the semen to aid in sperm motility. 

Figure 1. The prostate in the male reproductive system. (Adapted from the National Library of Medicine.)
In addition to its secretory function, the prostate also transports substances found in the blood from the basal side of the prostate into the lumen. In addition to nutrients and electrolytes, the prostate may then be exposed to the components of the man’s environment and to the various foods and chemicals he ingests, some of which may be carcinogenic. As the gateway to the testicle, the prostate is the site of significant inflammation caused by environmental exposure to bacteria and viruses over the course of a man’s life. The following section will discuss how exposure to certain environmental agents can cause PCa.

Figure 2. Diagram of the Zones of the Prostate.\textsuperscript{5}
CHRONIC INFLAMMATION and HALLMARKS OF PROSTATE CANCER

Extensive research into the origins of PCa has made it clear that PCa is the consequence of an accumulation of genetic and environmental insults on the prostate that manifest in old age. These insults cause chronic prostatic inflammation that can result in cancer. The causes of inflammation are myriad and varied. These range from infectious agents, such as from sexually and non-sexually transmitted bacteria or viruses; to dietary elements found in red or charred meats; to environmental or physical factors. Chronic inflammation of the prostate can lead to conditions called proliferative inflammatory atrophy (PIA), in which an increased fraction of proliferating cells exists within atrophic regions of the peripheral or transitional zones, or prostatic intraepithelial neoplasia (PIN), which exhibits molecular alterations similar to cancer. Both of these conditions are thought to be precursors to cancer.\(^7\)

The molecular hallmarks that cause PCa have been difficult to ascertain as few genes that strongly predispose men to developing PCa have been found. There have been several genetic aberrations identified in genes affecting cell survival, proliferation, xenobiotic detoxification, and AR activity that may play a role in carcinogenesis or increase risk. These include GSTP1; PTEN, which is frequently lost in prostate cancer and is correlated with poor prognosis\(^8\); the tumor suppressor p53; the AR; SRD5A2, the gene that encodes 5α-reductase, which converts testosterone to the more potent dihydrotestosterone in the prostate; and the androgen-responsive TMPRSS2-ERG fusion gene.\(^9\)

SCREENING, DIAGNOSIS, and MONITORING

Men with prostate cancer have benefitted from screening and early detection methods that aid clinicians in diagnosing disease while still at a curable stage. The primary form of screening is in the form of serum PSA, which is an enzyme encoded by an androgen-responsive gene that was found to track with disease progression and to correlate with length of survival.\(^10\) While PSA screening has been a staple of PCa early detection and is largely responsible for
decreases in mortality due to prostate cancer, its reliability has been controversial, and worries of overdiagnosis and overtreatment have arisen. Because PCa is a rather slow-growing cancer, it is possible that men diagnosed with PCa later in life may have such indolent disease that there would never have been a clinical presentation and they would have remained asymptomatic even without treatment. Localized PCa treatment with surgery or radiation is associated with significant side effects that can compromise quality of life and would cause unnecessary suffering for men with indolent disease. One study estimated that the number needed to screen (NNS) to save one life at 1410\(^{11}\), while estimates for the number needed to treat (NNT) vary widely from an acceptable low of 5 to an unacceptable high of 48.\(^{11-13}\)

Difficulties with using PSA as a screening tool arise from the fact that increases in serum PSA can result from noncancerous conditions, such as BPH, trauma, or inflammation. The search for other biomarkers to be used in conjunction with PSA for monitoring continues. Other candidates have included PTEN loss, gene fusion, and long non-coding RNAs that are increased in prostate cancer tissue but not normal or BPH tissue, among others.\(^{14}\)

Diagnosis for prostate cancer is usually made on based on histology features of tissue acquired during a needle biopsy. Signs of cancerous tissue are breakdown of organ architecture resulting in gland infiltration, increased cell number, enlarged nuclei, mitotic figures, absence of basal cells, and upregulation of \(\alpha\)-methylacyl-CaA racemase (AMACR). Tissue biopsies are given a Gleason score, which is a method of grading the malignancy of the cancer. The score is a sum of the grade of the most prevalent cell and the most undifferentiated cell, each graded from 1-5. Gleason scores correlate with prognosis and can inform treatment decisions.\(^9\)

**TREATMENT STRATEGY\(^{15}\)**

PCa treatment regimen depends on the disease stage, age, overall health of the patient, and the philosophy of the treatment center. The many treatment options are listed in Table 1
(reproduced from ref. 13) according to stage of disease. Each treatment will be explained in detail in this section.

Watchful waiting and active surveillance

Watchful waiting and active surveillance are reserved for asymptomatic older patients with low grade, low volume cancer or patients with comorbidities where the potential benefits of treatment may not outweigh the risks of treatment-associated side effects. With watchful waiting, treatment is not immediately administered upon diagnosis but can be initiated to alleviate symptoms if cancer progresses. Active surveillance means treatment for localized cancer is delayed until there is evidence of local progression or Gleason grade migration. The purpose behind this is to spare the patient from treatment side effects while preserving the curability of the cancer.

Radical prostatectomy

Radical prostatectomy is the complete removal of the prostate. This procedure is for men with organ-confined disease who are in good health and can bear the trauma of surgery. Men who undergo radical prostatectomy risk incontinence and impotence.

Radiation therapy and radiopharmaceutical therapy

Radiation therapy may consist of external-beam radiation aimed at the prostate and/or surrounding tissue or brachytherapy in which a sealed radiation source is placed inside or next to the affected area. Radiopharmaceutical therapy is used for the treatment of bone metastases.

Hormonal therapy

Hormonal therapy is administered with the goal of subverting the androgen pathways that promote PCa growth by reducing the level of androgen available to activate those pathways. The main focus of hormonal therapy in PCa is the androgen receptor (AR) pathway, which is described in more detail below. Initially, the goal of hormone therapy is to reduce plasma testosterone levels. This can be achieved by surgical (bilateral orchiectomy) or pharmacological
castration (lutenizing hormone-releasing hormone, LHRH, agonists/antagonists). The side effects of these treatments include hot flashes, impotence, decreased libido, weight gain, fatigue and osteoporosis.

In addition to castration, patients may undergo direct blockade of AR with the use of AR antagonists or inhibitors of enzymes involved in the synthesis of adrenal or intratumoral androgens. These therapies will be discussed in detail in subsequent sections.

Hormonal therapy, its mechanisms of action, and modes of resistance are the main foci of the research described in this dissertation.
Table 1. Prostate cancer treatment strategies according to stage.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Standard Treatment Options</th>
</tr>
</thead>
</table>
| Stage I   | • Watchful waiting or active surveillance  
           | • Radical prostatectomy  
           | • External-beam radiation therapy (EBRT)  
           | • Interstitial implantation of radioisotopes                                                |
| Stage II  | • Watchful waiting or active surveillance  
           | • Radical prostatectomy  
           | • EBRT with or without hormonal therapy  
           | • Interstitial implantation of radioisotopes                                                |
| Stage III | • EBRT with or without hormonal therapy  
           | • Hormonal manipulations (orchietomy or luteinizing hormone-releasing hormone [LHRH] agonist)  
           | • Radical prostatectomy with or without  
           | • EBRT  
           | • Watchful waiting or active surveillance                                                   |
| Stage IV  | • Hormonal manipulations  
           | • Bisphosphonates  
           | • EBRT with or without hormonal therapy  
           | • Palliative radiation therapy  
           | • Palliative surgery with transurethral resection of the prostate (TURP)  
           | • Watchful waiting or active surveillance                                                   |
| Recurrent | • Hormone therapy                                                                         |
THE ANDROGEN RECEPTOR

ANDROGEN RECEPTOR STRUCTURE AND FUNCTION

AR is a member of the nuclear hormone receptor (NHR) family of transcription factors. AR is a modular protein consisting of a transactivation domain at the N-terminus (NTD) followed by a DNA-binding domain (DBD), a hinge region, and a ligand-binding domain (LBD) at the C-terminus (CTD) encoded by 8 exons by the AR gene located on Xq11-12. AR activity is regulated by androgen-binding to the LBD\textsuperscript{16,17}. In the prostate, testosterone is converted to the more potent androgen dihydrotestosterone by 5α-reductase. In the inactive state, AR resides in the cytoplasm bound to heat shock protein-90 (HSP90). Upon androgen-binding to AR, HSP90 dissociates, AR dimerizes, enters the nucleus, and regulates the expression of AR-specific genes, such as PSA, as well as metabolic, survival, and proliferation genes\textsuperscript{18}. Nuclear translocation is regulated by a bipartite nuclear localization signal (NLS) located in the DBD and hinge region that interacts with the importin α/β nuclear import complex.\textsuperscript{19,20}

Androgen-binding induces several intermolecular and intramolecular interactions in AR. The receptor folds over on itself allowing intramolecular binding between FxxLF-like motifs (\textsuperscript{23}FQNL\textsuperscript{27} and, with lower affinity, \textsuperscript{433}WHTLF\textsuperscript{437}) in the NTD and residues in activation function-2 (AF2) of the LBD. In other nuclear hormone receptors other than AR, this site within the LBD serves as a docking site for coactivator and corepressor proteins with FxxLF or LxxLL-residues. However, the AR NTD competes with these coregulators for this binding site, and interaction between the NTD and LBD stabilizes ligand-binding to AR and protects AR from degradation.\textsuperscript{21}
Homodimerization of AR is primarily mediated through the DBD. AR interacts with AR target genes at androgen response elements (ARE), which are inverted hexameric repeats spaced by three nucleotides, usually of the sequence 5’AGGTCANNTGACCT3’, although other sequences have been reported. The DBD is composed of two zinc-fingers, one that binds to one repeat of the ARE and the other that binds to a zinc finger on the other androgen-bound AR monomer. AR monomers bind in a head-to-head fashion on DNA, such that the LBD of each monomer also dimerize.

AR is unique from other members of NHR family, such as GR and ER, in that the transactivation activity resides primarily in the NTC, rather than the CTD. The NTD is comprised of two transactivation units (TAUs), TAU-1 and TAU-5 (amino acids 360-528), the latter of which is responsible for ligand-independent transactivation. These TAUs bind coregulators that modify the chromatin structure to increase or decrease access to the transcriptional machinery. So far, 170 AR coregulators have been identified. These include histone acetyltransferases (HATs), such as p300/CBP and p160/SRC, that open up chromatin structure and increase transcription of target genes as well as histone deacetylases (HDACs) that repress transcription and histone de/methyltransferases than can have either effect depending on context. Interesting, AR itself can also be a target for these enzymes with varying effects on its activity.

**ANDROGEN RECEPTOR IN DEVELOPMENT**

Androgens play key roles in embryonic development, puberty, fertility, and adult homeostasis. The actions of androgens are believed to be mediated solely through AR. Gender is determined by the presence of the testis-determining factor or sex-determining region Y protein on the Y chromosome. This factor leads to the expression of genes that precipitate the development of the testes. Secretion of T from the testes is responsible for the development for the prostate, seminal vesicles, and other organs of the male reproductive system.
Prior to puberty, males undergo a period called adrenarche, in which changes in the male body are driven by adrenal androgens, such as DHEA and ACTH, rather than testosterone synthesized from the Leydig cells in the testes. These changes are marked by an increase in pubic and underarm hair. Conversely, maturation associated with puberty is driven by the androgenic and anabolic actions of testosterone. Puberty is marked by the enlargement of the testes and penis, body hair growth, the enlargement of the larynx and deepening of the voice, increased bone and muscle mass, height, behavioral changes, and spermatogenesis.25

In the adult male and female, testosterone is important for accumulation of bone mass during puberty, largely through aromatization to estrogen and subsequent activity of the estrogen-activated estrogen receptor, and maintaining bone health in adults. Testosterone increases osteoblast (the cell that produce the bone matrix) cell survival and proliferation, which decreases bone resorption, increase formation, and helps maintain bone mass.26

NORMAL FUNCTION OF ANDROGEN RECEPTOR IN THE PROSTATE

Contrary to its role in prostate cancer, the AR in normal prostate epithelium inhibits proliferation and promotes differentiation. The mature prostate is comprised of basal, luminal, and a small percentage of neuroendocrine cells. Basal cells are AR-negative, androgen-independent, and less differentiated than luminal cells.27 Basal cells also exhibit high proliferative and low apoptotic fraction.27 Luminal cells, in contrast, are AR-positive, androgen-dependent, highly differentiated – expressing such differentiation markers as prostate specific antigen (PSA) and prostatic acid phosphatase (PAP) 27 – and have balanced proliferative and apoptotic indices.28 Prostate stem cell divide asymmetrically to renew the stem cell population and produce transient amplifying cells, which also have the ability to self-renew. Transient amplifying cells differentiate into luminal and neuroendocrine cells.27,28

TESTOSTERONE BIOSYNTHESIS
The most important androgen in the male is testosterone, so before describing the methods of castration or inhibiting androgen binding to AR, this section will detail testosterone biosynthesis. Testosterone is one of the steroid hormones synthesized from cholesterol.

Biosynthesis of testosterone begins in the hypothalamus. Gonadotropin-releasing hormone (GnRH) secretion from the hypothalamus causes the release of the gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH) from gonadotroph cells of the pituitary gland. Gonadotropins are dimeric hormones containing a common α subunit and a unique β subunit. The testes are the target tissue for both FSH and LH, but LH is responsible for testosterone production from the Leydig cells and will be the primary gonadotropin discussed going forward.29

GnRH binds to GnRH receptors on gonadotrophs. GnRH receptors are 7-transmembrane G-protein coupled receptors that activate phospholipase C upon ligand binding. Phospholipase C liberates inositol 1,4,5-trisphosphate (IP$_3$) from phosphatidylinositol 4,5-bisphosphate (PIP$_2$) on the inner leaflet of the plasma membrane. IP$_3$ binds to ligand-gated Ca$^{2+}$ channels on the endoplasmic reticulum (ER), releasing Ca$^{2+}$ into the cytoplasm and upregulating transcription and release of FSH and LH into the circulation.29,30

The LH receptor on Leydig cells is also a G protein-coupled receptors. Binding of LH to its receptor causes exchange of GDP for GTP on the α-subunit of the heterotrimeric G-protein. The GTP-bound α-subunit activates adenylate cyclase, which converts AMP to cAMP. cAMP goes on to activate protein kinase A, which phosphorylates the CREB transcription factor and stimulates the transcription of enzymes involved in testosterone synthesis.29 cAMP synthesis also results in the transport of cholesterol from the outer to the inner mitochondrial membrane, the first step in testosterone synthesis.30

Testosterone is synthesized in response to acute as well as chronic LH stimulation. Acute testosterone synthesis is characterized by rapid transport of cholesterol to the inner mitochondrial
membrane, whereas chronic synthesis is regulated at the transcriptional level of the steroidogenic enzymes. The steroidogenic acute regulatory protein (StAR) plays a critical role in the transport of cholesterol from the outer to inner mitochondrial membrane. However, not much is known about its mechanism of action. Subsequent steps in the testosterone synthesis pathway consists of a series of reduction-oxidation reactions carried out by two classes of enzymes: cytochrome P450s (CYP) and hydroxysteroid dehydrogenases (HSD).

In the inner mitochondrial membrane, CYP450 side chain cleavage (P450scc) carries out three reactions, each requiring a molecule of oxygen and NADPH. As a result of these reactions, the C22-C20 bond of cholesterol is cleaved to produce the 21-carbon pregnenolone. Although P450scc does receive electrons from the mitochondrial transport chain, it is believed that the mitochondrial environment is needed for completion of these reactions rather than the chain itself. After side chain cleavage, pregnenolone diffuses to the endoplasmic reticulum (ER) where testosterone synthesis is completed.

In the ER, progrenolone is converted into the hormone progesterone by 3β-HSD. 3β-HSD converts progrenolone from a Δ5-3β hydroxysteroid to a Δ4-3 ketosteroid. This reaction requires the cofactor NAD as an electron acceptor. The third and fourth reactions are catalyzed by CYP17 (or 17α-hydroxylase). C17 is first hydroxylated to 17α-hydroxyprogesterone followed by C17-C20 bond cleavage to form androstenedione. Each reaction requires one molecule of oxygen and NADPH. Finally, in another oxygen and NADPH-requiring reaction, the C17 bond is reduced by 17β-HSD to form testosterone. Testosterone can further be metabolized to dihydrotosterone (DHT), the most potent, naturally-occurring androgen, by 5α-reductase, using NADPH as an electron donor or aromatized to estrogen by aromatase DHT can be further metabolized to the much weaker androgen 3α-androstenediol by 3α-hydroxysteroid dehydrogenase.
Testosterone accounts for over 90% of circulating androgen, but the pituitary gland also releases adrenocorticotropic hormone and stimulates the synthesis and release of androgens from the adrenal gland, accounting for the remaining 10% of circulating androgen 33.

**ANDROGEN DEPRIVATION THERAPY and TOTAL ANDROGEN BLOCKADE**

In 1941, Dr. Charles Huggins, widely considered the father of hormonal therapy in PCa, observed that the activity of serum acid phosphatase activity was strongly correlated with metastatic cancer and that acid phosphatase activity was decreased with castration and estrogen injections and increased by injections of testosterone. From his studies, he concluded that PCa was a hormonally responsive disease 34 and laid the foundation for androgen deprivation therapy (ADT). Subsequent treatment of men with advanced PCa by castration or estrogen injection proved efficacious. Huggins was awarded the Nobel Prize in Physiology and Medicine in 1966 for his contributions to the treatment of PCa. 35,36

ADT is achieved by the use of LHRH agonists. Although patients can initially experience tumor flare, the increase in agonist eventually results in LHRH receptor internalization and T synthesis downregulation. Patients who progress on ADT, said to then have castration-resistant prostate cancer (CRPC), have a couple hormonal treatment options remaining. To achieve total androgen blockade, abiraterone, a CYP17 inhibitor, can be used as a second-line therapy to block the synthesis of adrenal and intratumoral androgens 37 or androgen-AR binding can be directly blocked by antagonists such as enzalutamide 38. Much of the research in this dissertation will focus on resistance to enzalutamide. Therefore, the following sections will detail the discovery of enzalutamide as well as currently known mechanisms of resistance.

**ENZALUTAMIDE**

In spite of the various means that AR can signal in a castrate setting, it was apparent that many of these mechanisms converged upon AR and that the expression of this molecule was crucial for PCa cell survival, as indicated by microinjection experiments showing that inhibition
of signaling by an AR specific antibody decreased cell viability.\textsuperscript{39} Therefore, it was still reasonable to seek out a therapy targeting AR but that could overcome known resistance mechanisms.

Enzalutamide was discovered by Dr. Charles Sawyers in 2009. Sawyers sought an antiandrogen that could inhibit AR signaling in spite of AR overexpression or mutations in the ligand-binding domain, alterations that result in resistance to bicalutamide and flutamide. Sawywers overexpressed AR in LNCaP cells, which possesses a mutation in the LBD allowing ligand promiscuity, and in subsequent compound screens discovered enzalutamide. Enzalutamide exhibits no agonist activity and reduces AR nuclear translocation, DNA-binding, and co-activator recruitment.\textsuperscript{38}

In a phase I/II study of enzalutamide in patients, enzalutamide decreased PSA levels and inhibited androgen binding to androgen as evidenced by a FDHT-PET scan uptake study. This same study showed that patients without previous chemotherapy experienced greater response as measured by PSA than patients who had previously received chemotherapy,\textsuperscript{40} which led to separate phase III trials of enzalutamide in these different populations. In both studies, patients treated with enzalutamide fared significantly better than control patients. Both studies were halted and control patients were offered enzalutamide. The risk of death for patients on enzalutamide decreased by 29% and 37% and overall survival was improved by approximately 2 and 5 months for chemotherapy-naïve and chemotherapy-treated patients, respectively, compared to controls.\textsuperscript{41,42} Enzalutamide was approved by the FDA for the treatment of CRPC in 2012.\textsuperscript{43}

**RESISTANCE**

The mechanisms by which PCa cells evade hormonal therapy are varied and are still being elucidated. Some of the proposed mechanisms of persistent AR signaling include crosstalk with other growth factor pathways, AR amplification, increased sensitivity to castrate androgen levels\textsuperscript{44}, and mutations in AR resulting in ligand promiscuity and the ability for cancer
cells to use much less potent androgen metabolites or even antagonists such as flutamide and bicalutamide as agonists to activate AR. In recent years, an isoform of AR, referred to as ARV7 (or AR3), truncated after the DBD was discovered and was found to be strongly associated with resistance to enzalutamide and abiraterone. Similarly, the glucocorticoid receptor (GR) has been suggested as another means of circumventing total androgen blockade. The following sections will discuss the ARV7 and GR and their possible roles in resistance.

THE ANDROGEN RECEPTOR SPLICE VARIANT, ARV7, IN CASTRATION-RESISTANT PROSTATE CANCER

*Molecular Biology of ARV7*

ARV7 was discovered by two independent groups at Johns Hopkins and University of Maryland. Hu et al. discovered seven different AR splice variants truncated after exon 3 and therefore missing the ligand-binding domains. AR, ARV1, and ARV7 expression was increased in CRPC patients relative to hormone-naïve patients, but only increased expression of ARV7 was associated with post-surgical biochemical failure, i.e. rebound of previously low PSA levels, whereas ARV7/AR ratio, AR, or ARV1 expression did not reliably predict recurrence.

ARV7 is truncated after exon 3 and spliced to a cryptic exon 3 (CE3), which is found in intron 3 of the full length AR premRNA. Because ARV7 is truncated after exon 3, it lacks the second half of the bipartite NLS, which raises the question of how ARV7 nuclear localization occurs. Chan et al. showed that K629 and R631 in CE3 of ARV7 align with K629 and R631 in the hinge region of full length AR and that alanine mutation of these residues decreases nuclear localization but does not completely exclude ARV7 from the nucleus as it does full length AR. These results show that the two basic residues in the position of the canonical AR NLS in ARV7 are necessary for complete ARV7 nuclear localization but that there are other mechanisms by which ARV7 is localized to the nucleus that are distinct from full length AR. Furthermore, a ligand-regulated AR nuclear export signal (NES) has been located in the LBD and in the absence
of the NES, the remaining domains are nuclear. Because ARV7 has no LBD and therefore no NES, it stands to reason that although a mechanism exists for ARV7 to be transported into the nucleus, there is no mechanism, known at this time, for ARV7 to be exported from the nucleus.

Unlike full length AR, ARV7 nuclear localization and transcriptional activity is unregulated by androgen and is constitutively active in both androgen-depleted and replete conditions. While ARV7 regulates expression of normally androgen-responsive genes, such as PSA, there is evidence that ARV7 more strongly regulates a different set of genes associated with a proliferative phenotype compared to the gene subset regulated by full length AR, which is more associated with a differentiated phenotype. However, Li et al. found that ARV7 regulates a subset of the same genes that AR, not a different set of genes affiliated with proliferation. Li et al. showed the discrepancy may lie in how differentiation genes are regulated versus pro-proliferative genes. Whereas expression of genes like PSA continually increased with increasing DHT or ARV7 expression, M-phase genes behaved biphasically. The studies by Hu et al. were conducted at 1 nM R1881, and it is possible that at this concentration, M-phase genes would not be upregulated or even repressed and that ARV7 expression in these studies was at a stimulatory level.

ARV7 transcript levels are downregulated and upregulated by androgen and the antiandrogen enzalutamide, respectively, in different models of prostate cancer. Liu et al. demonstrated that the rate of ARV7 transcription is coupled to the rate of AR transcription – replete conditions decrease the AR transcription rate while deplete conditions increase the rate of AR transcription. As splicing outcomes are dependent on transcriptional rate due to the alterations in the loading of splicing factors onto the transcriptional machinery, an increased AR transcriptional rate leads to increased expression of ARV7. Liu et al. further showed that the change in splicing outcome is due to increased recruitment of splicing factors to the AR transcript in enzalutamide-treated VCaP cells and not a change in splicing factor expression. Splicing factor
recruitment was tracked to three regions of AR corresponding to the 3’, 5’, and 3’ exon-intron junctions of exon 3, cryptic exon 3b, and exon 4, respectively. While enzalutamide treatment elicited increased recruitment to the exon 3 and exon 4 splice sites, similar recruitment was not observed at cryptic exon 3b in LNCaP cells, which is consistent with the low expression of ARV7 in this cell line. Furthermore, Liu et al. identified intronic and exonic splicing enhancers (ISE and ESE) within or near cryptic exon 3b that, when mutated, specifically decrease expression of ARV7 and not full length AR. Splicing factors hnRNP1 and U2AF65 bind the ISE, and ASF/SF2 binds the ESE, interactions that are hindered by mutation of the splicing elements. Although enzalutamide did not change expression of splicing factors, siRNA-mediated inhibition of ASF/SF2, U2AF65, and hnRNP1 decreased expression of ARV7 with slight increases or decreases in AR expression.49

While ARV7 regulation by androgen in VCaP and LN95 is similar, the expression of ARV7 in 22Rv1 is more robust, with protein expression almost equivalent to full length AR, and less perturbed by hormonal changes. This behavior has been attributed to a tandem duplication of a region containing exon 3 in 22Rv1 that occurred during recurrence of a regressed CWR22 tumor in a castrated mouse, from which the 22Rv1 cell line was derived. This duplication was later reproduced and suggested the existence of breakpoint regions occurring between cryptic exon 2b and exon 3 and between cryptic exon 3b and exon 4. These regions are found within long interspersed nuclear regions, which are common sites of DNA breaks. Clinically, duplication of this region was only found in metastases of CRPC patients, not in primary tumors or the normal tissue of CRPC patients. It was noted by these authors, that therapies targeted at the cellular splicing machinery would be futile in patients that ARV7 stemming from this regional duplication.50 Interestingly, recurrence of the CWR22 has also resulted in the CWR-R1 cell line. A 48 kB deletion in intron 1 in a subset of these cells harbor that also results in the expression of ARV7.47
ARV7 and clinical resistance to enzalutamide and abiraterone

ARV7 has been shown to mediate enzalutamide resistance in *in vitro* assays and to be strongly associated with clinical resistance. siRNA knockdown of ARV7 in the 22Rv1 cell line not only resensitizes cells to androgen depletion and enzalutamide, knockdown of ARV7 further sensitizes cells to the stimulatory effects of androgen.\textsuperscript{47} The real question, however, is how reflective these models are of clinical resistance to small molecular AR antagonists and androgen synthesis inhibitors.

Probably the most important information to date cementing the relationship between ARV7 expression and enzalutamide resistance was gathered in a prospective trial of men treated with either enzalutamide or abiraterone. 62 men with CRPC were randomized to receiving either enzalutamide or abiraterone in addition to ADT. ARV7 status was determined prior to receiving treatment from mRNA harvested from circulating tumor cells (CTCs). The primary endpoint of this study was PSA response defined as a $\geq50\%$ decrease from baseline sustained for $\geq4$ weeks. Remarkably, by these criteria, no ARV7$+$ men responded to either enzalutamide or abieraterone. ARV7$+$ men also had worse response in secondary endpoints, having a shorter PSA and clinical or radiographic progression-free survival and overall survival. However, while ARV7 positivity was sufficient to predict response in this study, ARV7 negativity did not predict response. Only 55\% and 53\% of ARV7$-$ patients treated with abiraterone or enzalutamide, respectively, responded to these drugs. This result is not necessarily surprising as there are other known mechanisms of resistance to androgen therapy, as described above, that may apply here.\textsuperscript{51}

Of the 62 men in the study, 18 (30\%) were ARV7+. Some of the men had previously been treated with enzalutamide or abiraterone before entering the trial and receiving the other antiandrogen, i.e. men previously treated with enzalutamide were treated with abiraterone on the study and vice versa. Of the men previously treated with abiraterone or enzalutamide, 11 of 20 and 2 of 4, respectively, were ARV7+. Of those without prior abiraterone or enzalutamide
treatment, 1 of 11 and 4 of 27, respectively, were ARV7-. Combined, there were 13 men with previous treatment who were ARV7+ compared to 5 without previous treatment. Interestingly, there was no statistical difference between the response rate of ARV7+ and ARV7- men who had previously received abiraterone or enzalutamide, whereas there was a difference between ARV7+ and ARV7- men who had not previously received these treatments. This finding suggests cross-resistance between drugs and is again indicative of other resistance mechanisms.51

As ARV7 expression has been suggested to be a result of ADT49, the authors assessed the conversion of men from ARV7- to ARV7+. Of the 42 ARV7- men that gave ≥1 follow-up sample, 4 and 2 patients receiving enzalutamide and abiraterone, respectively, became ARV7+. There was no conversion in the other direction. While this result suggests causality between ARV7 status and antiandrogen treatment, the authors caution that the study did not have the statistical power or design to draw such a conclusion.51 In addition to whether abiraterone or enzalutamide can cause ARV7 expression in patients, the data also beg the question of whether ADT can also cause ARV7 expression and thus resistance to abiratone and enzalutamide.

ARV7 and clinical resistance to chemotherapy

It has been hypothesized that ARV7 could also mediate resistance to the microtubule inhibitor docetaxel. Docetaxel impairs the depolymerization required for nuclear trafficking, thereby inhibiting AR-regulated transcription. Since ARV7 is constitutively nuclear and would not require microtubule depolymerization, ARV7 could, in theory, mediate docetaxel resistance.52 However, a clinical study has shown that there was no difference in response to docetaxel between ARV7- and ARV7+ men.53 Another study did show, however, that men treated with abiraterone were less responsive to subsequent treatment with docetaxel. However, men on this study were not tested for ARV7 expression54, and again, there is no concrete clinical evidence that abiraterone causes ARV7 expression.
**Glucocorticoid Receptor in CRPC**

Although this dissertation will focus on ARV7 in CRPC, in recent years, the GR has emerged as a potential mechanism of enzalutamide resistance. Therefore, it seemed prudent to address this other possible mechanism of resistance and its possible interaction with ARV7. This section will highlight two papers that describe a potential role for GR in resistance to enzalutamide.

As enzalutamide was developed in LNCaP/AR cells, Sawyers used *in vivo* chronic exposure of these cells to enzalutamide to elicit resistance. A subsequent microarray of resistant LNCaP/AR tumors revealed that GR was one of the most upregulated genes relative to controls. GR and AR regulate overlapping gene sets, but not necessarily in the same fashion, and the microarray data was consistent with persistent blockade of AR-specific targets, but not of GR-specific targets, suggesting GR as a mechanism of circumventing AR blockade. Sawyers suggests a model of resistance in which selective pressure by AR blockade selects for GR+ cells followed by clonal expansion of such cells. This preclinical evidence coupled with clinical data that high basal expression of GR is correlated with poor response to enzalutamide and that antiandrogen-resistant tumors express GR strongly support GR as another mechanism of resistance. The clinical trial data about the use of GR agonists in PCa are mixed. Patients receiving corticosteroids in addition to enzalutamide on the AFFIRM trial fared worse than those not receiving enzalutamide, supporting the idea that GR can overcome androgen blockade. However, there are other trials that show CRPC patients have a clinically positive response to GR agonists, so ongoing use will require clinicians “appropriate clinical context” in which to use GR modulators.55

Data from the vander Griend lab supported many of these findings but added nuance to the GR story by examining the role of GR expression in a panel of PCa cell lines. Vander Griend also found that GR mRNA and protein were upregulated in response to *in vitro* castration and
chronic exposure to enzalutamide in LNCaP, LAPC4, and VCaP, but in xenograft models, only LAPC4 and VCaP showed a similar pattern. Resistant tumors showed an induction of AR prosurvival genes in the face of total AR blockade, one of which was SGK1, which is both an AR and GR target. A small molecule inhibitor of SGK1 made MDV3100 more effective, perhaps pointing to a new therapeutic target and strategy of targeting a downstream mediator of growth.\textsuperscript{56}

**PREVIEW OF RESEARCH**

The research presented in this dissertation will highlight three different projects concerning ARV7. The first is the origin of ARV7. It has already been established that ARV7 is a splice variant whose expression is due to gene fragment duplication or castration. I asked if the existence of ARV7 could be due to a dysfunctional mRNA surveillance system, specifically nonsense-mediated decay.

I next investigated the role of ARV7 in cell proliferation in \textit{in vitro} models of PCa. I next asked if ARV7 can biphasically regulate growth of certain PCa cell lines in the same manner that androgen does and if the mechanism of biphasic regulation is similar. Finally, I investigated the role of ARV7 in CRPC in several resistant models of PCa.

It is my hope that the findings presented in this dissertation will help push our collective understand of prostate cancer and especially of drug resistance further to alleviate the burden and suffering due to this disease.
CHAPTER 2. ANDROGEN RECEPTOR SPLICE VARIANTS ARE NOT SUBSTRATES OF NONSENSE-MEDIATED DECAY

INTRODUCTION

ARV7 is an androgen receptor (AR) variant that is truncated after the DNA-binding domain (DBD). Whereas nuclear translocation and transcriptional activity of wild type (WT) AR is regulated by androgens, the activity of ARV7 is uncoupled from such regulation and is active in both the presence and absence of androgens.\(^1\) It has been suggested that the transcriptional program of ARV7 drives proliferation, whereas full length AR is associated with a more differentiated phenotype\(^2\), although there is also evidence that genes regulated by ARV7 are simply a subset of those regulated by full length AR, which include both mitotic and differentiation markers.\(^3\) ARV7 has been associated with more progressive prostate cancer (PCa) and clinical resistance to the antiandrogen, enzalutamide, and the CYP17 inhibitor, abiraterone.\(^4\)

*In vitro* studies have established the expression of ARV7 as a mechanism of castration and enzalutamide resistance in a model of advanced PCa.\(^3\)

While the activity of ARV7 is not regulated by androgens, mRNA expression of the ARV7 can be. Androgen decreases expression of ARV7 mRNA and protein while castrate conditions and enzalutamide increase expression of ARV7 in the VCaP prostate cancer cell line.\(^5,6\) Previous studies have shown that gene transcription rates influence alternative splicing. In this model, castration and AR antagonism increases the rate of AR gene transcription, which favors formation of ARV7 and recruitment of splicing factors to the AR transcript, whereas androgen slows transcription and decreases expression of ARV7. Furthermore, knockdown of splicing factors ASF/SF2 and U2AF65 were found to reduce protein expression of ARV7 more than AR.\(^5\)

It was also noted that alternative splicing of AR results in the generation of pre-termination codon (PTCs)-bearing or nonsense transcripts and would suggest that mRNA
surveillance deficiencies or evasion could also be responsible for the expression of AR transcripts. Nonsense-mediated decay (NMD) is an mRNA surveillance pathway that degrades such transcripts before they are translated into truncated proteins, which could result in nonfunctional or toxic proteins. Nonsense transcripts are detected during translation. After mRNA splicing, the resulting exon junctions are marked by exon junction complexes (EJC). During translation, if ribosome-associated NMD proteins detect a stop codon upstream of an EJC, translation is terminated and nucleases are recruited to degrade the transcript.

To determine the role of NMD is in the expression of AR variants, we examined the expression and androgen regulation of up-frame shift protein 1 (UPF1), which is an RNA helicase required for NMD, as well as consequences of UPF1 knockdown on an NMD reporter and AR variant expression. We found that NMD in AR variant-expressing cells is able to recognize and degrade nonsense transcripts and that AR variant mRNA is not regulated by NMD.

**MATERIALS AND METHODS**

**Cell Lines**

CWR22 is an androgen-dependent cell line derived from a primary prostate tumor that is serially passaged in mice. 22Rv1 was derived from a CWR22 tumor that regressed after castration then recurred in this castrate setting (ATCC) The resulting cell line is androgen-independent, castrate-, and enzalutamide-resistant. LNCaP-95 and LAPC4-cr are the resistant cell lines derived in vitro in androgen-depleted media from LNCaP and LAPC4, respectively.

All media was supplemented with 1% Pen/Strep and 1% L-glutamine (Life Technologies, Grand Island, NY) as well the additional indicated supplements. RPMI 1640 and IMDM were purchased from Life Technologies. Fetal bovine serum (FBS) was obtained from Sigma-Aldrich (St. Louis, MO) and charcoal-stripped FBS from Gemini Bio-Products (West Sacramento, CA). LAPC4 cells were cultured in IMDM supplemented with 10% FBS and 1 nM R1881 (Sigma-Aldrich). 22Rv1 and LNCaP cells were cultured in RPMI 1640 supplemented with 10% FBS.
VCaP cells were cultured in DMEM (P/N: 30-2002, ATCC, Manassas, VA) supplemented with 10% FBS. LNCaP-95 cells were cultured in phenol-red free RPMI 1640 supplemented with charcoal-stripped FBS and B27 supplement (Life Technologies, Grand Island, NY). LAPC4-cr cells were cultured in phenol-red free IMDM supplemented with 10% charcoal-stripped FBS and B27 supplement.

*Nonsense-mediated decay reporter assay*

Nonsense mediated decay control and reporter pb510-HA-TBRβ-ZSG WT and pb510-HA-TCRβ-ZsG PTC+, respectively (hereon referred to as ZSG WT and ZSG PTC+, respectively) were generous gifts from Oliver Mühlemann (University of Bern, Bern, Switzerland) and were generated as previously described with the ZsG open reading frame (ORF) replacing the green fluorescent protein ORF. Cells were transfected in 10 mm tissue culture plates with the same amount of ZsG WT or ZsG PTC+ using FuGene HD Transfection Reagent (Promega, Madison, WI) according to the manufacturer’s instructions. After 48h, cells were trypsinized and evenly divided into two 60 mm plates for negative control (Silencer® Negative Control 1, Life Technologies, Grand Island, NY) or UPF1 siRNA transfection. Knockdown of UPF1 was achieved using the target sequence 5’-AAGAGAAUCGCCUACUUCACU-3’ (Silencer® Select siRNA, Life Technologies) using RNAiMax (Life Technologies), according to the manufacturer’s instructions. Knockdown was verified 48h post-transfection by semiquantitative qPCR. ZsG reporter mRNA and protein expression was determined by semiquantitative qPCR of the ZsG ORF (as described below) and flow cytometry.

*Reverse Transcription Quantitative Polymerase Chain Reaction*

Total RNA was harvested by RNeasy Plus Mini Kit (Qiagen, Venlo, Netherlands). On column DNA digestion of plasmid DNA was performed using RNase-Free DNase Set (Qiagen) for lysates from cells transfected with ZsG NMD reporter or control, according to the instructions found in the RNeasy Mini Kit handbook (Qiagen). Equivalent amounts of total RNA was used
between conditions for cDNA synthesis by the First Strand cDNA Synthesis Kit (Life Technologies). For the NMD reporter assay, a 1:100 or 1:1000 dilution of the resulting cDNA from 22Rv1 or LN95, respectively, was used for quantification of ZsG, whereas expression of UPF1 and TBP was quantified from the neat cDNA reaction. 2 µl of the diluted or neat cDNA reaction was used for semi-quantitative qPCR using iQ SYBR® Green Supermix and iCycler iQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA), using TATA-binding protein (TBP) as the reference gene. The following primers were used for qPCR:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZsG</td>
<td>5’-GACCGCTCTCTTCTGTTC-3’</td>
<td>5’-GAACCTTGACTCGTGACAT-3’</td>
</tr>
<tr>
<td>TBP</td>
<td>5’-CACGAACCACGGCCTGATT-3’</td>
<td>5’-TTTTCTTGCTGCCAGTCTGGAC-3’</td>
</tr>
<tr>
<td>UPF1</td>
<td>5’-GACCTGGGCTTAAACAAGA-3’</td>
<td>5’-TGAGCCGCATGTCTCTTAAC-3’</td>
</tr>
<tr>
<td>AR1</td>
<td>5’-CCATCTCTTGCTCTCGGAAATGTTATGAAGC-3’</td>
<td>5’-AGCTTCTGGTTGTCTCAGTGG-3’</td>
</tr>
<tr>
<td>ARV3</td>
<td>5’-AAGAGCCCGCTGAAGGATT-3’</td>
<td>5’-TTCTGTGACTCCCATTGGTG-3’</td>
</tr>
<tr>
<td>ARV71</td>
<td>5’-CCATCTCTGCTTCGGAATGTTATGAAGC-3’</td>
<td>5’-TTGGAATGAGGCAAGTCAGC-3’</td>
</tr>
</tbody>
</table>

The qPCR cycling protocol was as follows: 1 cycle at 95°C for 3 min, 35 cycles at 95°C for 30s, 60°C for 30s, 72°C for 45 s, followed by melt curve analysis from 65 to 95°C in 0.5°C increments for 5s each.
Statistical Analyses

Paired parametric t-tests and linear regression analyses were performed using GraphPad (La Jolla, CA).

RESULTS

NMD pathway is functioning in an AR variant-expressing cell line

To determine if the NMD pathway is functional in ARV7+ cell lines, we used a fluorescence-based reporter assay in which the ZsGreen1 (ZsG) ORF was inserted in-frame into a T-cell receptor β (TCRβ) exon upstream of an intron and the TCRβ stop codon (Figure 1A). After excision of the intron, the ZsG stop codon is >50 nucleotides upstream of an EJC, is recognized as a PTC, and is, therefore, a substrate for NMD. Inhibition of NMD by knockdown of UPF1 should increase expression of this reporter if the NMD pathway is functional.9

22Rv1, which expresses high levels of ARV7 protein3,5, was serially transfected with ZsG PTC+ followed by UPF1 or negative control siRNA. qPCR and flow cytometry of ZsG were used to determine reporter mRNA expression and fluorescence intensity, respectively. Expression of ZsG PTC+ increased significantly following knockdown of UPF1 (Figure 1B). These data indicate that the NMD pathway is intact in 22Rv1 cells.
**Figure 1. Nonsense-mediated decay assay in 22Rv1 cells.**

A) ZsG green fluorescent protein is inserted in-frame into a T-cell receptor β exon such that when the minigene is transcribed and spliced, the ZsG stop codon is located upstream of an EJC, is recognized as a PTC, and is subject to degradation by NMD. (Cartoon adapted from Paillusson et al., ref #9). B) siRNA inhibition of UPF1 in 22Rv1 cells causes a significant increase in reporter expression.

**AR variants are not NMD pathway substrates**

It was previously noted that alternative splicing of AR results in the creation of PTCs, which should render such transcripts vulnerable to degradation by NMD.¹ The clinically relevant and resistance-associated variant, ARV7, is not predicted to be a substrate of NMD (Figure 2A). However, ARV3, which has a PTC 124 nucleotides upstream of an exon-exon junction (Figure 2B) as well a proper stop codon in the terminal exon, has the structure of a canonical nonsense transcript, and we predicted that ARV3 would be a more potent activator of NMD than ARV7.

Because knockdown of UPF1 was sufficient to inhibit NMD in the above reporter assay, we again used UPF1 knockdown to investigate the effect of NMD inhibition on the expression of ARV3 and ARV7 in 22Rv1, LN95, and VCaP. ARV3 and ARV7 expression in 22Rv1 and VCaP was unaltered by knockdown of UPF1, but was statistically decreased in LN95 (Figure 2C).
Figure 2. Nonsense-mediated decay regulation of ARV3 and ARV7.

A) Structures of AR, ARV3, and ARV7. Stop codons are indicated by black triangles. B) Sequence structure of ARV3. Exon 2, cryptic exon 4, and exon 3 are highlighted in gray, green, and yellow, respectively. qPCR primers are underlined and the ARV3 stop codon is indicated in red font.

There are 124 nucleotides between the terminal nucleotide of the stop codon and the putative EJC at the junction of cryptic exon 4 and exon 3. Because the stop codon occurs >50 nucleotides upstream of an EJC, ARV3 would be expected to be a substrate of NMD. C) siRNA knockdown of UPF1 did not alter expression of ARV3 or ARV7 in 22Rv1 or VCaP but did decrease expression of ARV3 and ARV7 in LN95.

UPF1 not regulated by androgen

If NMD is involved in the adaptive cellular response to androgen that causes decreased expression of ARV7, it would be expected that expression of NMD regulators, like UPF1, would be increased in the presence of androgen causing an increase in ARV7 mRNA degradation. To that end, we investigated how UPF1 expression changes with androgen.
As expected, the expression of AR and ARV7 significantly decreased in 22Rv1, LN95, and VCaP with androgen. However, UPF1 expression did not change significantly in LN95 or VCaP, but 22Rv1 cells showed a statistical decrease in UPF1 expression (Figure 4).

![Graph showing expression levels of AR, ARV7, and UPF1 in different cell lines.](image)

**Figure 3. Androgen regulation of AR, ARV7, and UPF1 expression.**

AR and ARV7 expression are decreased by 1 nM R1881. UPF1 expression in LN95 and VCaP was unaffected by androgen, but expression of UPF1 was significantly decreased by androgen in 22Rv1 cells.

---

**No significant difference between UPF1 expression between sensitive and resistant cell or correlation between AR variant and UPF1 expression**

Conversion of cells to a castration- or enzalutamide-resistant phenotype is frequently accompanied by increased expression of AR and/or de novo expression of ARV7\(^{1,10}\). Although, NMD is functional in AR variant(+) cell lines, we examined UPF1 expression in sensitive, resistant, variant(-), and variant(+) cell lines for a correlation between resistance or variant status and UPF1 expression. Decreased UPF1 expression between sensitive parent cell lines and the resistant derivatives or between variant(-) and variant(+) cell lines would suggest that a dysfunctional NMD pathway could be characteristic of a resistance, such that significant amounts of a PTC-bearing transcript could evade regulation by NMD.

As expected, AR mRNA was significantly increased from LAPC4 to LAPC4-cr. There was a trend toward increased expression from CWR22 and 22Rv1, but this difference did not
reach statistical significance. AR expression between LNCaP and LN95 was stable (Figure 4). Also, as expected, ARV7 expression in CWR22 and LAPC4 was barely detectable and low in LNCaP. Resistant derivatives of CWR22 and LNCaP expressed many times more ARV7 than the parental cell lines (Figure 4). While expression of ARV7 in LAPC4-cr was statistically higher than that of LAPC4, expression was still barely detectable. ARV3 expression followed similar patterns for CWR22, LNCaP, and associated cell lines. Whereas AR, ARV3, and/or ARV7 expression was significantly different between sensitive and resistant lines, UPF1 expression was stable.

Figure 4. Expression of UPF1 in prostate cancer cell lines.

AR expression is statistically increased in resistant cell line LAPC4-cr relative to the sensitive parent cell line, LAPC4. There was no statistical difference in AR expression between LNCaP and CWR22 and its resistant derivatives, LN95 and 22Rv1, respectively. Both ARV3 and ARV7 expression are increased between CWR22 and 22Rv1 and between LNCaP and LN95. UPF1 expression is unchanged between sensitive and resistance cell lines.
Finally, we asked if UPF1 was in any way correlated to expression of ARV3 or ARV7 using linear regression analysis of ARV3 or ARV7 v. UPF1. If diminished expression of UPF1 was correlated with increased expression of ARV3 or ARV7, we would expect a negative slope. Linear regression analysis revealed a significant deviation from a slope of zero for ARV7 v. UPF1 in CWR22 and LAPC4-cr as well as for ARV3 v. UPF1 in LAPC4-cr and LAPC4 (Figure 5). However, all of these analyses reveal positive relationships, i.e. the more UPF1, the more AR variant, which is not what one would predict if UPF1 was a negative regulator of these variants. Also, these cell lines express very low levels of ARV3 and ARV7, so it is unclear what this finding means, if anything at all. The remaining cell lines showed no significant deviation from a slope of zero, indicating no correlation between ARV3 or ARV7 and UPF1 expression.

DISCUSSION

We investigated whether alterations in the functionality of the NMD mRNA surveillance pathway could be involved in the progression from a sensitive to resistant PCa phenotype. Resistant PCa is often marked by increased expression of AR and/or expression of the ARV7 AR variant, which is clinically associated with resistance to enzalutamide and abiraterone. It had previously been noted that alternative splicing of AR results in the generation of PTCs, which
should activate the NMD pathway and would suggest that the existence of the AR variants is due
to either the dysfunction or the evasion of this pathway.\textsuperscript{1} We investigated whether NMD
dysfunction is an additional marker of resistance.

First, we used an NMD reporter assay to show that NMD is functional in ARV7+ cells,
recognizing nonsense transcripts and degrading them before translation into protein. Next, we
showed through knockdown of UPF1 that neither ARV3 nor ARV7 are substrates for NMD and

---

**Figure 5. Linear Regression Analysis of ARV3 and ARV7 v. UPF1**

Correlation between expression levels of ARV3 or ARV7 vs. UPF1 was determined by linear
regression analysis. Solid lines indicate linear regression, and dashed lines indicate confidence
interval bands. Significant deviations from slopes of zero (indicated by p-values < 0.05) were
found in CWR22R (ARV7 v. UPF1), LAPC4 (ARV3 v. UPF1), and LAPc4-cr (ARV3 v. UPF1 and
ARV7 v. UPF1).
that unlike ARV7, the expression of UPF1 is not androgen-regulated. Finally, we showed, through linear regression analysis, that UPF1 expression is not correlated with ARV7 expression in cells that express high levels of ARV3 or ARV7 transcript and/or protein and that expression of AR variants is likely not dependent upon UPF1.

Upon further examination of the position of the stop codon in the coding sequence of ARV7, it is not expected that this stop codon would activate the NMD pathway. NMD is activated when a PTC is located >50 nucleotides upstream of an exon-exon junction, as marked by EJC's, and has at least one intron downstream\textsuperscript{11}. \textsuperscript{12} The ARV7 stop codon occurs in the terminal exon, so no intron or EJC would be located downstream of this codon.

However, inclusion of cryptic exon 4 into the ARV3 transcript does create a canonical NMD target. The resulting ARV3 PTC is 124 nucleotides upstream of an exon-exon junction site and has two introns downstream, which should trigger degradation of the transcript during translation. There are several mechanisms by which putative nonsense transcripts evade NMD. In-frame translation reinitiation at a start codon downstream from the PTC can also cause nonsense transcript evasion of NMD.\textsuperscript{15} In addition to another PTC in cryptic exon 4 two codons downstream from the 5'-'most PTC, there is an in-frame start codon 9 codons downstream from the 5'-most PTC in cryptic exon 4 followed by a proper stop codon in the terminal exon. Therefore, it is possible that termination reinitation is mechanism of NMD evasion for ARV3.

Other mechanisms of evasion are not predicted to apply to ARV3. Transcripts with nonsense codons that are too close to the AUG translational start site are not regulated by NMD due to temporal interference of ribosomal initiation, elongation, and termination and loading of associated factors, as has been noted with β-globin transcript variants. Fewer than 18-20 codons between the AUG and nonsense codons is thought to render nonsense transcripts resistant to NMD.\textsuperscript{13} There are over 600 codons between the AUG and nonsense codons in ARV3, so evasion of NMD is not expected to be an issue of proximity. Positioning of poly(A)-binding protein
(PABPC1) in close proximity to the PTC also suppresses degradation of nonsense transcripts. Positioning of PABPC1 15 codons downstream from the PTC increased a NMD-resistant transcript by 3-fold, whereas when the distance between the PTC and the PABPC1 binding site was tripled, the transcript was only increased 1.5-fold. The 3’UTR of ARV3 is >500 codons downstream of the PTC, so it is unlikely that a short 3’UTR is the mechanism of NMD resistance.

While the ARV3 transcript is able to evade mRNA surveillance, it is unknown whether ARV3 produces a protein. If there was a corresponding protein, the putative ARV3 protein would be truncated in the DBD, would probably be severely, functionally impaired either as a constitutive transcription factor or dominant negative protein, and was determined most likely to be clinically irrelevant and was not further pursued in studies about AR variants and their role in prostate cancer.

NMD escape and dysfunction can be important contributing factors to the etiology of a disease. Many of the rules for NMD targets are based on studies on β-globin. Nonsense mutations in β-globin can cause a blood disorder called β-thalassemia. Mutations in exons 1 and 2 of β-globin trigger NMD and result in a mild form of β-thalassemia in people heterozygous for these mutations. However, nonsense mutations in exon 3, the terminal exon, do not trigger NMD, produce a dominant negative form of β-globin, and a form of β-thalassemia that can lead to severe anemia. Mutations in the components of NMD can also result in upregulation NMD targets. Recently, mutations in intronic and exonic splicing enhancer regions of UPF1 were identified in the malignant regions of pancreatic adenosquamous carcinoma tumors. The resulting alternatively spliced UPF1 mature mRNA produced deletions of the helicase domain or C-terminal domain, which is phosphorylated during NMD. Impaired NMD correlated with increased expression of a long form of the tumor suppressor p53.

There are 9 different proteins that are involved in NMD. We focused on UPF1 in this study because UPF1 is required for NMD to go forward and inactivating mutations in UPF1 are
sufficient to inhibit NMD\textsuperscript{7,18} and cause a decreased rate of decay of nonsense transcripts. Furthermore, inhibition of NMD by translation inhibitors, such as cycloheximide or emetine,\textsuperscript{19} or knockdown of UPF 1\textsuperscript{20} is a proven method of identifying nonsense targets. NMD proteins play many roles\textsuperscript{21}, and we cannot rule out the possibility that some are affected and may mediate the rise of resistant cancer. However, at this time, we do not expect that deficiencies in the NMD pathway are involved in expression of AR splice variants.

REFERENCES


CHAPTER 3. THE ANDROGEN RECEPTOR SPLICE VARIANT, ARV7, IN BIPHASIC ANDROGEN RESPONSE

INTRODUCTION

In the normal prostate, the androgen receptor (AR) promotes differentiation and suppresses proliferation of prostate epithelial cancer cells. During progression from a normal to malignant phenotype, prostate luminal cells express increased AR levels, and the AR signaling pathway undergoes a conversion from a tumor suppressive to an oncogenic pathway that promotes proliferation and survival. An example of this oncogenic switch is the change from a repressor to a stimulator of Skp2, which promotes cell cycle progression. Furthermore, the oncogenic activity of AR is further aided by the fusion of the 5’ untranslated region of the TMPRSS2 gene, which contains androgen response elements (AREs), to the ERG oncogene. This gene fusion is one of the most frequent chromosomal rearrangements found in prostate cancer (PCa). Targeting of the AR pathway, achieved by pharmacological or surgical castration, AR antagonists, or androgen synthesis inhibitors, exploits the reliance of PCa cells on AR and causes tumor regression. This is an initially effective strategy that ultimately fails due to myriad mechanisms resulting in castration-resistant prostate cancer (CRPC).

However, androgen can still be growth suppressive to some PCa cells due to the biphasic effect of androgen on PCa cells, stimulating proliferation at low concentrations while inhibiting growth at high concentrations. Tissue culture media supplemented with 10% hormone-replete fetal bovine serum (FBS) contains a castrate level of testosterone that is converted and concentrated intracellularly to 10 nM dihydrotestosterone (DHT), which supports optimal growth in tissue culture. Addition of the synthetic androgen, R1881, pushes growth into the inhibitory phase of the biphasic androgen growth curve. A few mechanisms account for these observations. First, AR behaves as a DNA replication licensing factor. AR levels cycle from low to high from early G1 to M-phase, respectively. Proteosomal degradation of AR at the end of M-
phase removes AR from origins of replication and permits the next round of division. Stabilization of AR against degradation by androgen in FBS-containing media results in continued presence of AR at origins of replication and prevents proper licensing with causes cell cycle arrest in the subsequent S-phase.\(^4,5\) Furthermore, while prostate differentiation markers like prostate specific antigen (PSA), continually increase with increasing androgen, genes expressed during or just prior to M-phase are regulated in a biphasic manner.\(^6\) For example, the AR coactivator, dopa decarboxylase, is also regulated biphasically\(^7\), raising the possibility that a different cast of coactivators and repressors interacts with AR at varying androgen concentrations.

These observations formed the rationale for a pilot study in which men with CRPC were initially treated with a combination of etoposide and supraphysiological doses of testosterone followed by testosterone alone. This study demonstrated clinical efficacy in some patients as measured by PSA and radiographic regression.\(^8\)

The AR variant, ARV7, a ligand-binding domain deletion splice variant of AR, is localized to the nucleus and regulates androgen-responsive genes independently of androgen. ARV7 mediates resistance to the AR antagonist, enzalutamide, in the 22Rv1 cell line. Expression of ARV7 is correlated with PCa progression and clinical resistance to enzalutamide and the testosterone synthesis inhibitor, abiraterone.\(^6,9,10\) ARV7 regulates M-phase genes in a biphasic manner similar to androgen-activated full length AR, with low ARV7 protein levels stimulating expression of M-phase genes and higher levels inhibiting expression.\(^6\)

In this paper, we investigated the consequences of overexpressing ARV7 in the enzalutamide-sensitive cell lines, LNCaP and VCaP, in tissue culture media supplemented with charcoal-stripped serum (CSS), which is depleted of steroid hormones, as well as FBS. We show that ARV7 expression displays similar dichotomous behavior on cell growth in CSS and FBS that
is similar to what is observed with treatment with low to high concentrations of R1881 and that the growth-inhibitory effects of ARV7 in FBS is not generalizable to all PCa models.

METHODS

Cell Lines

All media was supplemented with 1% Pen/Strep and 1%L-glutamine (Thermo Fisher Scientific, Grand Island, NY) as well the additional indicated reagents, unless otherwise specified. RPMI 1640 and IMDM were purchased from Thermo Fisher Scientific. FBS was obtained from Sigma-Aldrich (St. Louis, MO) and CSS from Gemini Bio-Products (West Sacramento, CA). LAPC4 cells were maintained in IMDM supplemented with 10% FBS and 1 nM R1881 (Sigma-Aldrich). 22Rv1 and LNCaP cells were maintained in RPMI 1640 supplemented with 10% FBS. VCaP cells were maintained in DMEM (ATCC, P/N: 30-2002, Manassas, VA) supplemented with 10% FBS. LN95 cells were maintained in phenol-red free RPMI 1640 supplemented with 10% CSS and B27 serum supplement (Thermo Fisher Scientific, Grand Island, NY). LAPC4-cr cells were cultured in phenol-red free IMDM supplemented with 10% CSS and B27 serum supplement.

Plasmids, RNA Interference, and Transfections

pcDNA3.1-ARV7 was a generous gift from Dr. Jun Luo (Brady Urological Institute, Johns Hopkins University, Baltimore, MD) and was generated as previously described.11 pcDNA3.1-AR was generated by inserting wild type AR cDNA at the EcoRI restriction site of the pcDNA3.1 multiple cloning site using the In-Fusion Cloning kit (Clontech Laboratories, Mountain View, CA).

Cells were transfected with pcDNA3.1-ARV7, pcDNA3.1-AR, or the empty vector with Fugene HD Transfection Reagent (Promega, Madison, WI) according to the manufacturer’s instructions. LNCaP cells transfected with varying amounts of ARV7 were transfected with a total of 7 µg DNA in a 60 mm dish with the empty vector providing the balance of DNA.
Transfection of ARV7 (target sequence 5’- GUAGUUGAGAGUAUCAUGA-3’), Silencer® Select siRNA, Thermo Fisher Scientific, AR (target sequence 5’- UCAAGGAACUCGAUCGUAU-3’), Silencer® Select siRNA, Thermo Fisher Scientific, or negative control (Silencer® Negative Control 1, Thermo Fisher Scientific) siRNA was achieved using Continuum transfection reagent (Gemini Bio-Products) or, for harder to transfect cells (LN95 and LNCaP), Lipofectamine RNAiMax (Thermo Fisher Scientific) according to the manufacturer’s instructions. LNCaP transfected with increasing concentrations of AR siRNA were transfected with a total of 5 nM siRNA with negative control providing the balance of siRNA.

Successful transfection was verified by immunoblot or quantitative reverse transcription polymerase chain reaction (qRT-PCR) as described below.

**Immunoblotting**

Cells were harvested by trypsin and washed in PBS. Cells were counted then lysed in 10 mM Tris-HCl, 10 mM NaCl, and 1% NP40 at a concentration of 100,000 cells per 10 µL lysis buffer for 15 min on ice. Following addition of sample loading buffer, proteins were resolved on SDS-PAGE gel, transferred to a PVDF membrane, and blocked with 5% w/v powdered milk in 1% TBS-T wash buffer. The following antibodies were diluted as indicated in blocking buffer and incubated overnight with the membrane at 4°C: AR Antibody (N-20) (Santa Cruz Biotechnology, Dallas, TX) 1:2,000; ARV7 (A&G Precision Antibody, Columbia, MD) 1:1,000; total GR (P/N: 12041, Cell Signaling Technology) 1:2,000; and β-actin (P/N:A5441, Sigma Aldrich) 1:20,000. The appropriate HRP-conjugated secondary antibody was diluted in blocking buffer to 1:4000, 1:2000, 1:4000, and 1:40,000, respectively, and incubated with the membrane for 1 hour at room temperature. Protein bands were visualized on ECL film after incubation with HRP substrate.

**qRT-PCR**

42
Total RNA was harvested by RNeasy Plus Mini Kit (Qiagen, Venlo, Netherlands). cDNA synthesis was carried out with the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). An equal amount of RNA was used between control and experimental conditions for each experiment. 1 µL of the cDNA reaction was used for semi-quantitative qPCR with iQ SYBR® Green Supermix and iCycler iQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA), using TATA-binding protein (TBP) as the reference gene. The following primers, synthesized by Integrated DNA Technologies (Coralville, IA), were used for the polymerase reaction:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBP</td>
<td>Fwd 5’-CACGAACCACGGCACTGATT-3’</td>
<td>Rev 5’-TTTCTTGCTGCCAGTCTGGAC-3’</td>
</tr>
<tr>
<td>AR10</td>
<td>Fwd 5’-CCATCTTGTCTTCTCGGAAATGTATGAAGC-3’</td>
<td>Rev 5’-AGCTTCTGGTTGTCTCCTCAGTG-3’</td>
</tr>
<tr>
<td>ARV710</td>
<td>Fwd 5’-CCATCTTGTCTTCTCGGAAATGTATGAAGC-3’</td>
<td>Rev 5’-TTTGAATGAGGGAAGTGTCAGCCTTCT-3’</td>
</tr>
<tr>
<td>PSA</td>
<td>Fwd 5’-CAGGTGTAGACCAGAGTGTTTC-3’</td>
<td>Rev 5’-CTGTGTCCTCAGAGAAATGTAG-3’</td>
</tr>
<tr>
<td>CCNA2</td>
<td>Fwd 5’-TCCTCCTTGGAAAGCAAACA-3’</td>
<td>Rev 5’-GGGCATCTTCTAGCCTATATT-3’</td>
</tr>
<tr>
<td>CDCA5</td>
<td>Fwd 5’-AAGGATGTAGGGAGCCTTCTAGT-3’</td>
<td>Rev 5’-GCCCTATCAACCTGGCATT-3’</td>
</tr>
<tr>
<td>ZWINT</td>
<td>Fwd 5’-ATCCCTGGGATCTCTTCAGTT-3’</td>
<td>Rev 5’-CCCAGCTCCTGTCATGTTAT-3’</td>
</tr>
<tr>
<td>UBE2C</td>
<td>Fwd 5’-CCTCATGATGTCTGGCGATAA-3’</td>
<td>Rev 5’-CCTCAGGTCTTCATATACGTTCCT-3’</td>
</tr>
</tbody>
</table>
For samples transfected with pcDNA3.1-ARV7 or empty vector, an on-column DNA digest was performed using RNase-free DNase1 (Qiagen), according to the manufacturer’s instructions. cDNA from that reaction was diluted 1:100 before ARV7 expression analysis. For all other transcripts, analysis was performed from the neat solution. A cDNA synthesis control reaction excluding the reverse transcriptase enzyme was performed to ensure complete digestion.

Androgen deprivation and hormonal treatment

Cells were androgen deprived as previously described¹² before the start of hormonal treatment assays in CSS. Prior to overexpression of AR in LAPC4, which are cultured in media containing R1881, cells were subjected to the androgen deprivation protocol with a final incubation in FBS-containing media in an effort to remove R1881 before the hormonal assay. Cells were treated for the indicated lengths of time. R1881 was diluted in ethanol and diluted to the indicated concentration. Enzalutamide (Selleckchem, Houston, TX) was dissolved in DMSO to a stock concentration of 100 mM and further diluted in DMSO to the indicated concentration.

Cell Viability Assay

Cells were plated in quadruplicate in a 96-well format in 100 μL of media. Cells were treated on day 0 of the assay by adding 100 μL of a 2X concentration of the indicated drugs for a final concentration in the well of 1X in 200 μL. For baseline (day 0) measurement, cell number was measured by MTT assay (Promega) according to the manufacturer’s instructions. Briefly, 15 μL MTT dye was added to each well and incubated in the dark at 37°C for 4 hours. The reaction was stopped with 100 μL solubilization buffer and allowed to incubate overnight at room temperature on a shaker. Absorbance at 570 nM with a background subtraction of 650 nm was used to determine cell number from a standard curve generated for each cell line. For all other days, 100 μL of media were carefully removed from each well using a multichannel pipette so as not to disturbed adherent cells before performing the MTT assay.
RESULTS

Hormonal and ARV7-mediated growth characteristics of VCaP and LNCaP in CSS and FBS

First, we characterized the growth of LNCaP cells in response to the synthetic androgen, R1881, and the AR antagonist, enzalutamide, in CSS and FBS. In CSS, growth of LNCaP (Figure 1, CSS) was stimulated by R1881 while growth in response to 1 µM enzalutamide was static. Curiously, the combination of the AR agonist and antagonist also stimulated overall growth. In FBS, LNCaP (Figure 1, FBS) proliferation is inhibited by both 1 nM R1881 and 1 µM enzalutamide. Again, the combination of R1881 and enzalutamide elicits a similar level of growth as vehicle-treated cells. These effects were reproducibly observed.

Next, we studied the effect of ARV7 overexpression on growth of the AR variant negative LNCaP in CSS and FBS. It has previously been shown that ARV7 expression stimulated growth of LNCaP in CSS.\textsuperscript{13} Here, we show that in FBS overexpression of ARV7 has
the opposite effect in FBS with a growth-inhibitory effect on LNCaP that is similar to R1881 and enzalutamide (Figure 2).

**Figure 2. ARV7-mediated growth inhibition of LNCaP in FBS**

Overexpression of ARV7 in LNCaP inhibits growth in FBS.

**Effect of R1881, enzalutamide, and ARV7 on PSA and M-phase gene expression**

We next examined the effect of R1881 and enzalutamide on expression on the androgen-responsive differentiation marker, PSA, and genes associated with M-phase when cells are incubated in CSS and FBS. We hypothesized that expression of PSA would increase with R1881 and decrease with enzalutamide and that enzalutamide would inhibit R1881-stimulated expression in both CSS and FBS, whereas M-phase gene expression would parallel the cell growth observed in each type of serum. All transcripts were analyzed from the same sample.

We analyzed the M-phase-associated genes CCNA2, CDCA5, UBE2C, and ZWINT. These genes were found to be regulated biphasically in LNCaP cells in CSS by both androgen and ARV7. In addition, UBE2C was previously identified as a gene that may be selectively upregulated by AR in CRPC cells compared to sensitive cells and more upregulated by ARV7 than by full length AR. CCNA2 is a ubiquitously expressed cyclin that activates CDK1 and CDK2. CDCA5 is involved in binding of sister chromatids during G2. UBE2C is a member of...
the E2 ubiquitin-conjugating family and is necessary for cyclin degradation and cell cycle progression.\textsuperscript{17} The exact role of ZWINT in cell division is unknown, but it is known that the encoded protein is involved in proper kinetochore function.\textsuperscript{18}

Figure 3. Androgen-Regulated Expression of PSA in LNCaP in CSS and FBS.

A) In both CSS and FBS, androgen-stimulated expression of PSA is inhibited by enzalutamide in LNCaP. B) Androgen- and enzalutamide-mediated effects on M-phase-associated gene expression in LNCaP parallel growth patterns when grown in CSS. For LNCaP grown in FBS, expression is consistently and considerably inhibited by R1881 across all genes analyzed, whereas enzalutamide and the combination of R1881 and enzalutamide only slightly inhibit expression relative to controls.

As expected, LNCaP PSA expression was upregulated by R1881, downregulated by enzalutamide, and intermediate when cells were treated in both CSS and FBS (Figure 3A).
Expression of all M-phase genes in LNCaP grown in CSS was perfectly reflective of growth responses in each condition (Figure 3B, left panel). Expression of all genes was increased with R1881, unaffected by enzalutamide, and comparable to that of R1881-treated cells.

Figure 4. ARV7-regulated Expression of PSA and M-phase genes in CSS and FBS.

A) In CSS, ARV7 overexpression stimulates PSA expression and has a biphasic effect on M-phase gene expression, with expression peaking at 2 μg ARV7. B) In FBS, ARV7 overexpression does not affect expression of PSA but does significantly decrease expression of CCNA2 and UBE2C.
The relationship between gene expression and growth in FBS was less clear (Figure 3B, right panel). In FBS, all M-phase genes were markedly downregulated by R1881 relative to the control. Enzalutamide decreased expression of all M-phase genes but was not as potent a downregulator of gene expression as R1881. Expression of genes, when cells were treated with the combination of R1881 and enzalutamide, was much higher than with R1881 alone but only slightly lower than vehicle-treated cells, consistent with growth characteristics in response to treatment with both the agonist and antagonist.

We then asked what affect ARV7 overexpression had on expression of these genes in both types of serum. ARV7 overexpression increases expression of PSA in CSS but not FBS (Figures 4A and 4B, respectively, middle panels). Consistent with the effect of ARV7 on growth, ARV7 has a biphasic effect on M-phase gene expression in CSS but significantly decreased expression of CCNA2 and UBE2C (Figure 4A and 4B, respectively, bottom panels).

**Influence of full-length AR protein level on growth in FBS**

Previously, it was shown that high expression of AR in the AR-negative PCa cell line, PC3, resulted in growth inhibition, whereas low expression had a minimal effect.\(^5\) Here, we show in AR-positive cell lines that partial knockdown of AR in LNCaP abrogates the growth suppressive effects of androgen. Partial knockdown of AR (Figure 5A) decreased growth of LNCaP cells in FBS (Figure 5B). As shown above, LNCaP treated in FBS are growth-inhibited by 1 nM R1881 (Figure 5C). When treated with R1881, cells with partially knockdown AR, were no longer growth-inhibited by R1881 (Figure 5D, E). Importantly, cells with decreased AR still experienced net growth, so the lack of an androgen effect is not due to AR knockdown already causing maximal growth inhibition. We also wondered if increased AR expression would cause androgen-mediated inhibition in the normally androgen-stimulated LAPC4 cell line in FBS. LAPC4 transfected with vector showed marginally significant increased growth in response to
R1881 relative to the vehicle. Overexpression of AR abrogated this effect but did not inhibit growth (Figure 6).

**ARV7-mediated resistance in various prostate cancer cell lines**

Li et al. has shown that expression of ARV7 renders the 22Rv1 insensitive to androgen deprivation, enzalutamide-mediated growth inhibition, and androgen-mediated growth stimulation and that that knockdown of ARV7 restores all effects. Here, we examine a panel of

![Arv7](image)

**Figure 5. Partial knockdown of AR abrogates androgen-mediated growth inhibition in FBS**

A) AR was partially knockdown using increasing concentrations of siRNA. B) siRNA knockdown of AR in LNCaP suppresses growth in FBS. Addition of R1881 suppresses growth of cells treated with 0 nM AR siRNA (C), but has no impact on growth when AR levels are decreased (D and E).

ARV7(-) and ARV7(+) enzalutamide-resistant cell lines to understand mechanisms of resistance in commonly used *in vitro* models of PCa. The following studies were conducted in CSS after hormonal depletion.
Figure 6. Overexpression of AR abolishes stimulatory effect of androgen on LAPC4 growth.

LAPC4 transfected with vector were marginally statistically stimulated by R881 in FBS relative to vehicle-treated cells (p=0.05). Androgen treatment has no effect on LAPC4 overexpressing AR (p=0.5). Differences between AR transfected cells treated with vehicle or R1881 and 1 μM enzalutamide were statistically significant (p = 0.03 and 0.03, respectively), as well as the difference between vehicle and 1 nM R1881/1 μM enzalutamide (p=0.004).

LAPC4-cr is a castration resistant derivative of LAPC4, created by chronic passaging in CSS. LAPC4-cr cells are ARV7 and GR(-) but show increased expression of AR relative to LAPC4 (Figure 7A). LAPC4 were growth-stimulated by R1881, inhibited by androgen-depleted media and enzalutamide, and 1 μM enzalutamide was sufficient to completely antagonize the effects of 1 nM R1881 (Figure 7B). LAPC4-cr, however, grew robustly in all conditions.

The LN95 cell line is derived from LNCaP, also by chronic exposure to androgen-depleted media. Oddly, LNCaP expresses more AR protein than LN95 (Figure 8A). However, LN95 is ARV7(+) but GR(-). As shown above, LNCaP growth in CSS is stimulated by R1881
Figure 7. Castration-resistant growth of LAPC4-cr

A) The castration-resistant derivative of LAPC4, LAPC4-cr, expresses increased AR protein but no ARV7 or GR. B) LAPC4 growth is sensitive to hormonal manipulations, while LACPC4-cr grow robustly in all conditions.

and inhibited by androgen-depleted media and enzalutamide. However, the combination of R1881 and enzalutamide has no effect on growth.

LN95 cells grow robustly in all conditions tested. Because LN95 AR expression is less than that of LNCaP and are GR(-), we hypothesized that ARV7 was the mechanism of resistance in this cell line. However, when ARV7 was inhibited by siRNA, LN95 growth behavior was unchanged, even though a high level of ARV7 knockdown was maintained for the duration of the assay (Figure 8B).
Figure 8. Castration-resistance of LN95 is not mediated by ARV7.

A) AR protein levels are decreased from LNCaP to its castration-resistant derivative, LN95, which expresses the AR variant, ARV7. Both cell lines are GR(-). B) Sustained knockdown of ARV7 does not resensitize LN95 to AR blockade.

CWR22 cells are derived from a primary human PCa tumor that is serially passaged in immunocompromised mice. Upon castration of CWR22-tumor-bearing mice, subsequent regression, and recurrence, the 22Rv1 was established and is maintained in vitro. CWR22 are both AR(+) and GR(+) but ARV7(-), whereas 22Rv1 express markedly higher levels of AR and GR and de novo expression of ARV7 (Figure 9A). As such, this presented an opportunity to assess the contribution of GR and ARV7 to resistance in the same cell line. As demonstrated by Li et al., 22Rv1 treated with ARV7 siRNA are resensitized to growth suppression by androgen-depletion and enzalutamide and more sensitive to stimulation by androgen. We also showed that due to the presence of GR in 22Rv1, treatment with GR agonist, dexamethasone, is not only able
to overcome growth suppression by androgen depletion or antagonism but stimulates growth to a greater degree than R1881 in ARV7 siRNA-treated 22Rv1 (Figure 9B).
Figure 9. Castration-resistant 22Rv1 are resensitized by ARV7 inhibition but restimulated by activation of GR pathway

A) Castration of CWR22 tumor-bearing mice and subsequent recurrence of a regressed tumor resulted in the 22Rv1 cell line, which is shows increased AR and GR levels and de novo expression of ARV7. B) siRNA knockdown of ARV7 resensitizes 22Rv1 to androgen deprivation and AR antagonism. However, GR agonist, dexamethasone, allows 22Rv1 to grow in CSS and in the presence of enzalutamide even in the absence of ARV7.
From these data, we conclude that while ARV7 can support growth in a castrate setting, expression of ARV7 does not necessarily indicate mechanistic involvement in resistance. Furthermore, cells that are mechanistically reliant on ARV7 for resistance can circumvent ARV7 downregulation by coexpression of GR.

**ARV7 supports growth of 22Rv1 in FBS**

We next asked why 22Rv1, which expresses a high level of full length AR and ARV7, is not growth inhibited in FBS. To address this question, we knocked down ARV7 in media containing CSS and FBS and assayed for differences in growth and gene expression. Above, we showed that ARV7 supports growth in CSS. We hypothesized M-phase gene expression of ARV7(+)-22Rv1 cells would reflect the growth supportive role of ARV7. We also hypothesized that in androgen-replete FBS, ARV7 knockdown would have no effect on growth because full length AR would be sufficiently activated to replace ARV7.

Expression analysis of M-phase genes in 22Rv1 incubated in CSS showed that ARV7 significantly increased expression of all four genes analyzed as well as PSA expression (Figure 10A). By contrast, there was no significant difference in the expression of CCNA2, CDCA5, or ZWINT between negative control and ARV7 siRNA-treated cells in FBS. UBE2C did display a slight but statistical increase in the absence of ARV7 (Figure 10B). This is in contrast to LNCaP, in which growth and M-phase gene expression increased and inhibited in CSS and FBS, respectively, by overexpression of ARV7. Even though gene expression was largely unaffected, knockdown of ARV7 still resulted in statistically decreased growth in FBS relative to negative control-treated cells (Figure 11).
Figure 10. ARV7-mediated gene expression in 22Rv1 in CSS and FBS

Gene expression from cells treated with ARV7 siRNA, and are therefore ARV7(-), are shown in black, and those treated with negative control siRNA, and are therefore ARV7(+), are shown in gray. ARV7 significantly increases expression of PSA in A) CSS (p < 0.0001) and B) FBS (p = 0.03) and significantly upregulates expression of CCNA2, CDCA5, UBE2C, and ZWINT (p < 0.001 for all transcripts) in CSS. UBE2C exhibits a small but statistical decrease in expression in response to ARV7 expression in FBS, but all other M-phase transcripts are unchanged.
Figure 11. ARV7 inhibition in 22Rv1 significantly affects growth in FBS.

Although 22Rv1 are able to grow robustly in FBS without ARV7, the AR variant does provide a net growth advantage to 22Rv1.

DISCUSSION

In this study, we compared the effect of ARV7 and androgen on growth and gene expression in the LNCaP PCa cell line. We also characterized the role of ARV7 in various in vitro models of resistant PCa, paying special attention to the ARV7(+) 22Rv1 cell line, which was shown to rely on ARV7 castration resistance.

In CSS, androgen-mediated growth in LNCaP is inhibited by enzalutamide. We expected that the combination would elicit intermediate growth, but it was instead stimulatory to a similar to that of R1881-treated cells. At the moment, we do not have an explanation for this observation. However, our search of the literature did not yield contradictory data. In one study, 1 nM DHT-mediated LNCaP growth was inhibited by 5 µM enzalutamide, an agonist:antagonist ratio of 1:5000\(^1\), suggesting that although enzalutamide administered at a concentration 1000X that of R1881 was sufficient to inhibit androgen-stimulated growth of all other enzalutamide-sensitive or sensitized cell lines in this study, a greater concentration may be needed for LNCaP and VCaP cells. This could be due to the high expression of AR in these two cell lines.
In FBS, however, R1881 and enzalutamide were both inhibitory to growth while cells treated with the combination demonstrated a similar growth response to vehicle-treated cells. Androgen-mediated growth inhibition in FBS is consistent with the biphasic effect of androgen on growth. Cells have titrated AR expression to support optimal growth given the level of endogenous androgen found in media supplemented with 10% FBS. Additional androgen increases active AR to an inhibitory level, whereas AR antagonists drop active AR levels to a suboptimal level. The combination of the AR agonist and antagonist, however, returns cells to optimal growth even though both treatments are individually inhibitory because enzalutamide antagonizes the inhibition by R1881 (or, to look at it another way, R1881 reverses the growth inhibition by enzalutamide), returning the concentration of active AR to an optimal level. This was further supported by the decreased inhibitory effect of R1881 on LNCaP treated with AR siRNA and the insensitivity to androgen in LAPC4 cells overexpressing AR. However, AR protein knockdown or expression in these respective cell lines only caused androgen insensitivity, not stimulation or inhibition, respectively, suggesting that there is more to androgen-mediated inhibition than AR content.

Our data shows that M-phase-associated gene expression in LNCaP grown in CSS almost perfectly parallels growth patterns whereas gene expression of LNCaP in FBS is less reflective of growth. These data could be indicative of cell line-specific hormonal responses, differential growth-response curves for each gene, or varying influences of each gene on growth. From a practical research standpoint, our data may signify why most hormonal studies are conducted in hormone-depleted media using the LNCaP cell line.

ARV7 behaves like ligand-bound AR in that it is nuclear and can regulate AR-responsive genes. A previous study showed that ARV7 is also similar to ligand-bound AR in that increasing expression of ARV7 also has a biphasic effect on M-phase genes in CSS. Similar to androgen-mediated effects, we did observe ARV7-mediated growth inhibition in FBS. M-phase
genes were similarly regulated. However, we were also unable to detect an effect of ARV7 expression on PSA in FBS.

We investigated the role of ARV7 in enzalutamide resistance in several *in vitro* models of prostate cancer. The cell lines profiled in this study exhibited a wide range of resistance mechanisms. Before ARV7 and GR were discovered as possible mechanisms of resistance to androgen blockade, it was known that there are several other mechanisms by which PCa cell circumvent AR blockade. These include overexpression of AR coactivators or reduced expression of corepressors, ligand-independent activation by other growth pathways, mutations in the ligand-binding domain, and intratumoral steroidogenesis. The most common change between castration-sensitive and -resistant cancer is increased expression of AR, either by increased transcription or multiplication of the AR gene. The LAPC4 cell line behaves like the “quintessential” PCa cell line. LAPC4 are growth-stimulated by androgen and even require the presence of 1 nM R1881 in FBS for optimal growth in tissue culture. LAPC4 respond predictably to both androgen depletion and enzalutamide. Long-term culture in CSS results in increased AR expression in the resulting LACP4-cr cell line although we cannot rule out the possibility that other mechanisms of resistance are in effect.

Of the cell lines profiled, LN95 is probably the most interesting. While we were able to confirm previous findings that ARV7 in 22Rv1 is sufficient for sustaining castration-resistant growth, our data show that LN95, which is also ARV7(+), grow robustly when ARV7 is inhibited by siRNA. LN95 are also GR(-) and charcoal-stripping removes glucocorticoids as well as androgens, so we do not predict the GR pathway to be the mechanism of resistance. Our data that LN95 AR expression is not increased relative to AR expression in the parental cell line, LNCaP, and is probably not a mechanism of resistance is consistent with previous findings showing AR message and protein levels are similar between the two cell lines. These results suggest that there may be some other factor required for ARV7-mediated resistance.
Perhaps one of the most important questions that need to be addressed is how well *in vitro* and *in vivo* models of resistant prostate cancer reflect clinical data. An initial clinical study showed that ARV7 expression as detected by mRNA in circulating tumor cells perfectly identified enzalutamide- and abiraterone-resistant patients and showed that ARV7(-) patients have ~50% chance of response. By protein expression, our data, in large part, agrees. Both 22Rv1 and LN95, which express high levels of ARV7, are enzalutamide-resistant, even though resistance is not mediated by ARV7 in LN95, and LAPC4-cr are ARV7(-) but enzalutamide-resistant. VCaP, which express barely detectable levels of ARV7 protein, are highly sensitive to androgen-depletion and enzalutamide (Figure 12).

Interestingly, VCaP expresses a high amount of ARV7 mRNA. The level of ARV7 in VCaP was sufficient for use in assay development in the aforementioned prospective enzalutamide study in which ARV7 was identified as a biomarker for resistance. The authors were able to detect ARV7 to a limit of 5 cells in 5 mL of blood. The discrepancy between
protein and mRNA expression explains why VCaP cells are not resistant to enzalutamide and grow so poorly in CSS and suggests that protein expression would be a more reliable biomarker, as mRNA expression could result in falsely labeling sensitive patients as resistant.

Targeting ARV7 is an area of intense interest in CRPC research.\textsuperscript{24,25} Upregulation of M-phase-associated genes in 22Rv1 cells expressing ARV7 in CSS further supports its role in resistance to AR blockade. Our data also show that even if cells can be resensitized to AR blockade through inhibition of ARV7, activation of other pathways can circumvent ARV7-targeted therapies as well. In addition to \textit{de novo} expression of ARV7, 22Rv1 cells upregulate expression of GR, which can restimulate growth of cells in the presence of ARV7 siRNA and AR antagonists. Corticosteroids are often administered to castration-resistant patients treated with chemotherapy as a part of a palliative regimen that may also have antitumor effects.\textsuperscript{26} In light of our data and data showing clinical association between GR expression and poor enzalutamide
response of resistant tumors, the consequences of coadministration of dexamethasone and other corticosteroids during therapy and a potential role for GR antagonists for treatment of resistant cancer should be carefully investigated.

Our finding that ARV7 knockdown in 22Rv1 decreases growth in FBS without affecting the expression of M-phase genes was surprising, but it suggests that full length AR may be able to compensate for decreased ARV7 expression in the regulation of these genes. It would also suggest that there are other genes that ARV7 specifically regulates that impact growth.

In conclusion, androgen and ARV7-mediated cell growth is highly dependent on cell-type and environmental context, with AR protein levels playing a major role. Furthermore, we found that ARV7-mediated resistance to AR blockade is complicated. ARV7 expression may only be a marker of resistance for some patients, whereas expression may actually mediate resistance in others. Additional molecular signatures will be required to distinguish the two populations.

REFERENCES


CHAPTER 4. CONCLUSION

In the preceding dissertation, I sought to build upon the current knowledge surround the role AR and its splice variant, ARV7, in CRPC.

While it is known that in some contexts ARV7 mRNA and protein expression is responsive to androgen levels and that ARV7 is a product of a splicing error or errors, much is still unknown about its origin. We asked if there was perhaps an error in mRNA surveillance mechanisms, particularly NMD, that could account for the high expression of this variant. We found that NMD appears to be functioning in ARV7(+) cell lines, that there is no significant difference between expression of the NMD mediator, UPF1 between sensitive and resistant cell lines, and that although there is some correlation in certain cell lines between UPF1 and AR variant expression, these relationships occur in sensitive cell lines and do not fit with the theory that UPF1 would be a negative regulator of AR variants. It was important to rule out NMD impairment as a possible mechanism of high AR variant expression so that the field of AR variant research can focus their attention elsewhere to elucidated the genesis, regulation, and perhaps targeting of splice variants for treatment of CRPC.

The second focus of this dissertation was on the biphasic nature of ARV7. Androgen has a biphasic effect on growth of LNCaP in CSS and completely inhibits growth in FBS. We showed that ARV7 displays similar behavior and that this behavior can, in part, be attributed to patterns in gene expression. This extended previous androgen-based observation made by Dr. John T. Isaacs to the constitutively active ARV7. We also did the important work of reproducing and confirming many previous findings. Given that molecular biology often suffers from the problem of reproducibility, the fact that we achieved many of the same results suggests that a solid groundwork has been laid upon which future research can be built. Beyond reproducing, we also showed that the role of ARV7 in resistant cancer is complicated, mediating resistance in some cell lines but not in others. This will be an important consideration going forward for
ARV7-targeted therapies, as additional markers for mechanistic involvement of ARV7 may be needed.

Additionally, future directions should be focused on understanding more about the biology of ARV7 and how it differs from that of AR. We also do not yet understand the molecular underpinnings, in terms of interaction with coregulators and specific AR-regulated genes of biphasic growth response regulated by either AR or ARV7 and why some genes are regulated biphasically and monophasically.

It is my hope that the research presented in this dissertation answered some important outstanding questions in AR research and helped direct the future research AR biology.
CURRICULUM VITAE

A. Seun Ajiboye

Date of birth: March 30, 1986

Location of birth: Raleigh, NC, USA

Education

August 2009 – Dec. 2015  Johns Hopkins School of Medicine, Baltimore, MD
Degree: PhD, Pharmacology

August 2004 – May 2008  Duke University, Durham, NC
Major: BS, Chemistry with Pharmacology Concentration
GPA: 3.3

Research Experience

August 2009 – exp. Dec. 2015  Pharmacology and Molecular Sciences Graduate Program
Denmeade Lab, Johns Hopkins School of Medicine, Baltimore, MD

Dissertation: The Androgen Receptor Splice Variant, ARV7, in Castration-Resistant Prostate Cancer: Molecular Origins, Biphasic Growth Regulation, and Resistance

August 2008 – July 2009  Cancer Research Training Award Fellowship
Figg Lab, Medical Oncology Branch, National Cancer Institute, Bethesda, MD

August 2007 – May 2008  Research Independent Study
Seewaldt Lab, Pharmacology and Cancer Biology Department, Duke University, Durham, NC

June – August  Howard Hughes Research Fellowship
2005  Keene Lab, Molecular Genetics and Microbiology Department, Duke University, Durham, NC

August 2007 – May 2008  Science Education Research Independent Study
Pharmacology and Cancer Biology Department, Duke University, Durham, NC

Leadership Experience

August 2009 – exp. Dec. 2015  Graduate student mentor (3 students)
Denmeade Lab, Johns Hopkins School of Medicine, Baltimore, MD

Jan. 2015 – June 2015  Tong Zhang Innovation Fellow
Thread, Baltimore, MD

January 2013 – 2015  Project Manager
Thread, Baltimore, MD

Publications

The androgen receptor splice variant, ARV7, is not a consequence of impaired nonsense-mediated decay. *Manuscript in preparation.*

Combination of protein synthesis inhibitor and androgen receptor antagonist, enzalutamide, is synergistic and reverses resistance in prostate cancer cell. *Manuscript in preparation."

The androgen receptor splice variant, ARV7, in biphasic androgen response. *Manuscript in preparation.*


**Presentations**

**Ajiboye AS**, Denmeade SR. Regulation of hormone receptor splice variants. Poster presented at: Molecular Therapeutics of Cancer Research Conference. 7th annual meeting of the Cancer Molecular Therapeutics Research Association; 2014 July 13-17; Pacific Grove, CA.

**Ajiboye AS**, Denmeade SR. A strategy for identifying small molecule and stapled peptide inhibitors of the amino-terminus of the androgen receptor. Poster presented at: Multi-Institutional Prostate Cancer Retreat. 5th annual meeting of the Prostate Cancer SPORE Program. Participating Institutions included: The Johns Hopkins University, The University of Michigan, Memorial Sloan-Kettering Cancer Center, and the Dana Farber Cancer Institute; 2012 March 19-21; Fort Lauderdale, FL.

**Honors and Awards**

**2007 – 2008**

Recipient of the National SMART Grant

Awarded by the federal government to undergraduates maintaining a minimum GPA of 3.0 in a science, math, or technology major

**2004 – 2008**

American Chemical Society Scholars Program

Four-year merit scholarship awarded to high school seniors from underrepresented groups pursuing an undergraduate degree in the chemical sciences

**2004**

National Achievement Scholarship

Scholarship awarded to select group of African-American high school seniors based on high performance on the PSAT/NMSQT

**2004**

Ronald McDonald House Charities/African-American Future Achievers Scholarship

Scholarship awarded to African-American high school seniors based on academic achievement and community involvement

**Memberships and Organizations**

American Society of Pharmacology and Experimental Therapeutics (2014 – present)